

MEETING

SCIENTIFIC ADVISORY BOARD

MARCH 14, 15, 16, 1973

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725CM CHALON

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

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January 23, 1973

Modified Application for New Grant No. 725CM

To: The committee comprising Drs. Gardner, Loosli and Sommers

Subject: Jack Chalon, M.D., Albert Einstein College of Medicine,
Bronx
Modified Application for New Grant No. 725CM
"Changes in Tracheobronchial Cytology"

History

An earlier application considered by the SAB in September, 1972 was deferred for further information. Dr. Chalon conferred with CTR staff on January 4, 1973, and as a result has submitted a detailed modification of his previous application.

This applicant has been supported since 1969. His pending request (No. 725CM) is for \$18,110. plus one additional year.

Documents Submitted (attached)

1. Letter (8 pages) dated January 10, 1973.
2. Application dated July 5, 1972 (This is the same application you have seen previously: the voluminous appendices are not included).

Comment:

A progress report was forwarded to you on August 9, 1972.

F.W.N.
F.W.N.

FWN/vr
Encl.

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ALBERT EINSTEIN COLLEGE OF MEDICINE
OF YESHIVA UNIVERSITY

1300 MORRIS PARK AVENUE, BRONX, N.Y. 10461 • CABLE:EINCOLUMED, N.Y.

DEPARTMENT OF ANESTHESIOLOGY

PHONE: (212) 430-2000

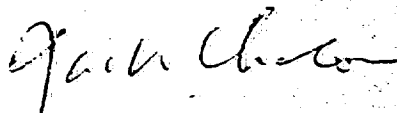
January 16, 1973

Robert C. Hockett, Ph.D.
Acting Scientific Director
The Council for Tobacco Research - U.S.A., Inc.
110 East 59th Street
New York, New York 10022

Dear Dr. Hockett:

I enclose the letter you requested at our meeting on January 4th
in relation to a two year extension of Grant #725.

Sincerely yours,



J. Chalon, M.D.
Assistant Professor

JC/kb
Enc.

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ALBERT EINSTEIN COLLEGE OF MEDICINE
OF YESHIVA UNIVERSITY

1300 MORRIS PARK AVENUE, BRONX, N. Y. 10461 • CABLE: EINCOLMED, N. Y.

DEPARTMENT OF ANESTHESIOLOGY

PHONE: (212) 430-2000

January 10, 1973

Drs. Sheldon C. Sommers and Robert C. Hockett
The Council for Tobacco Research-U.S.A., Inc.
110 East 59th Street
New York, New York 10022

Dear Drs. Sommers and Hockett:

I thank you for the interview which you granted me on January 4, 1973 in connection with the proposed extension of Grant number 725.

Following preliminary research work done in the last six months and after discussing the matter with you, I feel the following relevant points should be made with regards to the course of study to be followed in connection with the project entitled "Changes in tracheobronchial cytology;" and based on the following points:

1) During the period: October 1, 1969 to July 1, 1972 smears from 1,365 anesthetized women were analyzed in relation to nuclear position changes and goblet cell percentage variations during the menstrual cycle and the effect of old age, pregnancy and exogenously administered sex hormones on these cytologic changes.⁽¹⁾

"Cyclic cytomorphologic and cytochemical changes were noted in the epithelia of younger women but not in the epithelia of older women and males. During the proliferative phase of the menstrual cycle the nuclei of the ciliated cells were found mainly in the basal position. Following ovulation they ascended to mid-position, and as the secretory phase progressed they migrated towards the apex. After approximately the 25th day, increasing numbers of cells with basal nuclei were again noticed. Intracytoplasmic mucopolysaccharide content increased with the ascent of the nucleus. When cells with basal nuclei predominated once more, goblet cells proliferated concurrently, probably indicating metaplasia from ciliated cells with high mucopolysaccharide content."

It was assumed that these cyclic fluctuations might probably protect females from inhaled irritants and carcinogens.

2) In order to assess the effects of cigarette smoking on the cyclic variations described above, efforts were made to compare nuclear position variations and goblet cell percentage changes between women with different smoking habits. It was found that there were very little

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differences in that respect between non smokers and light and medium smokers. Heavy smokers, however, displayed generally increased goblet cell percentages and a less marked nuclear position variation during the menstrual cycle.

3) Damage to the components of the ciliated epithelial cells (cilia, end plate, nucleus and cytoplasm) was noted in several smears and appeared at first to be more marked in patients who had breathed dry anesthetic gases.⁽²⁾ A special study was conducted on healthy non smokers of both sexes using a point scoring system to assess damage numerically and it was found that dry gases produced significant cytologic damage if used for over an hour whereas gases with a 60% humidity at room temperature and fully humidified at body temperature did not, for periods in excess of three hours.

4) Cellular damage caused by dry anesthetic gases are not specific and similar changes are found between the various smoking groups. This is in accordance with histologic studies made by Auerbach et al⁽³⁾ who also describe increases in goblet cell percentages in smokers.

Based on the last two points, a pilot study was conducted on a group of 180 patients to try and correlate smoking habit with preoperative lung function tests, percentages of normal cytologic features at the onset of anesthesia and postoperative complication rate. In addition a special study subgroup was formed in which smoking habit was correlated with rate of decay of cytologic features between the onset and end of anesthesia when dry and humidified gases were used.

Preoperative lung function tests (figure 1) differed little between non smokers, light, medium and heavy smokers but there was a marked decrease in lung function in very heavy smokers. These results are similar to those obtained by other authors⁽⁴⁻⁶⁾. The correlation between preoperative lung function and postoperative complication rate was of the order of $R = 0.65$.

The percentage of normal cytologic features at the onset of anesthesia, on the other hand, varied markedly between the various smoking groups being highest in non smokers and lowest in very heavy smokers (figure 2). In this instance the correlation coefficient between intraoperative cytology (at the onset of anesthesia) and the rate of postoperative complications was 0.92, thus indicating a much closer relationship between these two factors.

The rate of cytologic decay during anesthesia (table 1) did not vary between light and non smokers and medium and heavy smokers if the gases were humidified, whereas when dry gases were used, light and non smokers fared best.

Considering the results obtained thus far, it is felt that a two year special study should be conducted to try and correlate the possible

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protective effects of female hormones with cytologic damage caused by smoking, preoperative lung function and postoperative complication rate.

In fact, if damage to the cells caused by smoking was found to vary with alterations in goblet cell percentage in younger women during the menstrual cycle and if this could be correlated with a decreased postoperative complication rate and improved lung function, our assumption that females are indeed protected by their sex hormones against bronchogenic carcinoma (and possibly obstructive lung disease) would be strengthened.

The following line of study is proposed:

1) To study a group of young female patients subdivided by smoking habit (amount and duration); all patients being about to undergo routine surgery under general endotracheal anesthesia.

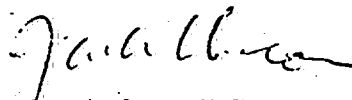
Investigation to include:

- a) Relevant history and required historical data
 - b) Preoperative lung function tests (Peak expiratory flow rate, FEV1 and FEV3)
 - c) Study of tracheobronchia smears obtained at the onset of anesthesia in relation to:
 - i) Nuclear position
 - ii) Percentage of goblet cells
 - iii) Calculation of the percentage of normal cellular features
 - d) Postoperative evaluation including the monitoring of temperature, examination of the chest (physically and Roentgenologically if needed) and blood gas analysis if relevant.
- 2) Controls to include older females and males of various age groups and of various smoking habits.
- 3) A special study on the possible protective effects of exogenous hormones and other steroids in patients receiving these drugs preoperatively or intraoperatively for therapeutical reasons.
- 4) A study to evaluate the resistance to dry anesthetic gases between various smoking groups of both sexes (cytology and postoperative follow up).
- 5) To continue the search for malignant cells appearing from time to time in smears and to arrange for the follow up of patients producing these cells.

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In addition as there seems to be a racial and environmental influence on the incidence of bronchogenic carcinoma (7) it may be advisable to repeat the original hormone study in Japan. Arrangements have already been made to put this into action should the Council feel that it would be of interest.

Sincerely yours,



J. Chalon, M.D.
Assistant Professor

JC/mm
enc.

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APPENDIX 1

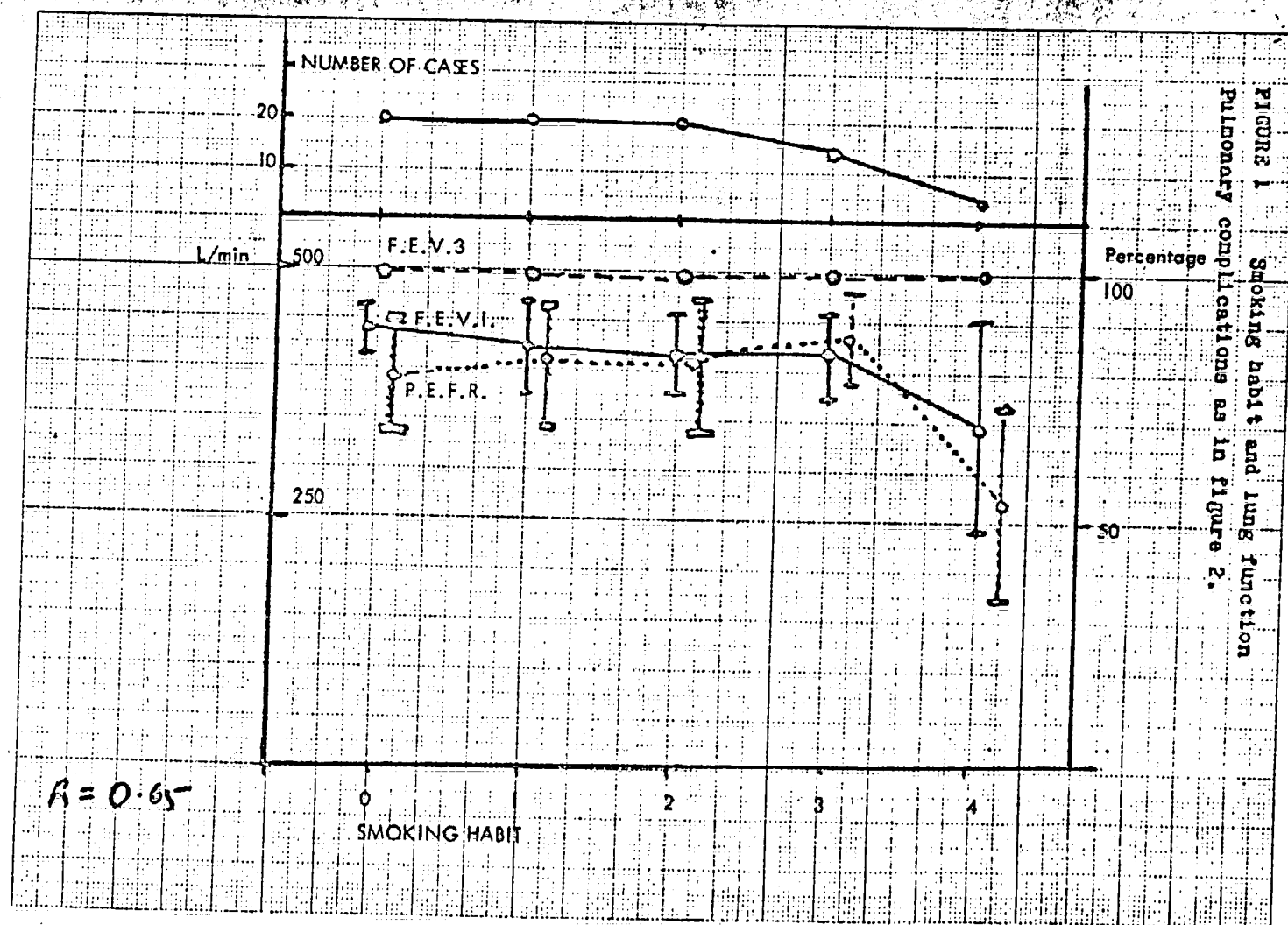
ABBREVIATIONS USED IN FIGURES 1 AND 2

Smoking Groups: 0 Non smoker
1 Light smoker (1-9 cigarettes a day)
2 Medium smoker (10-19 cigarettes a day)
3 Heavy smoker (20-29 cigarettes a day)
4 Very heavy smoker (over 30 cigarettes a day)

REFERENCES

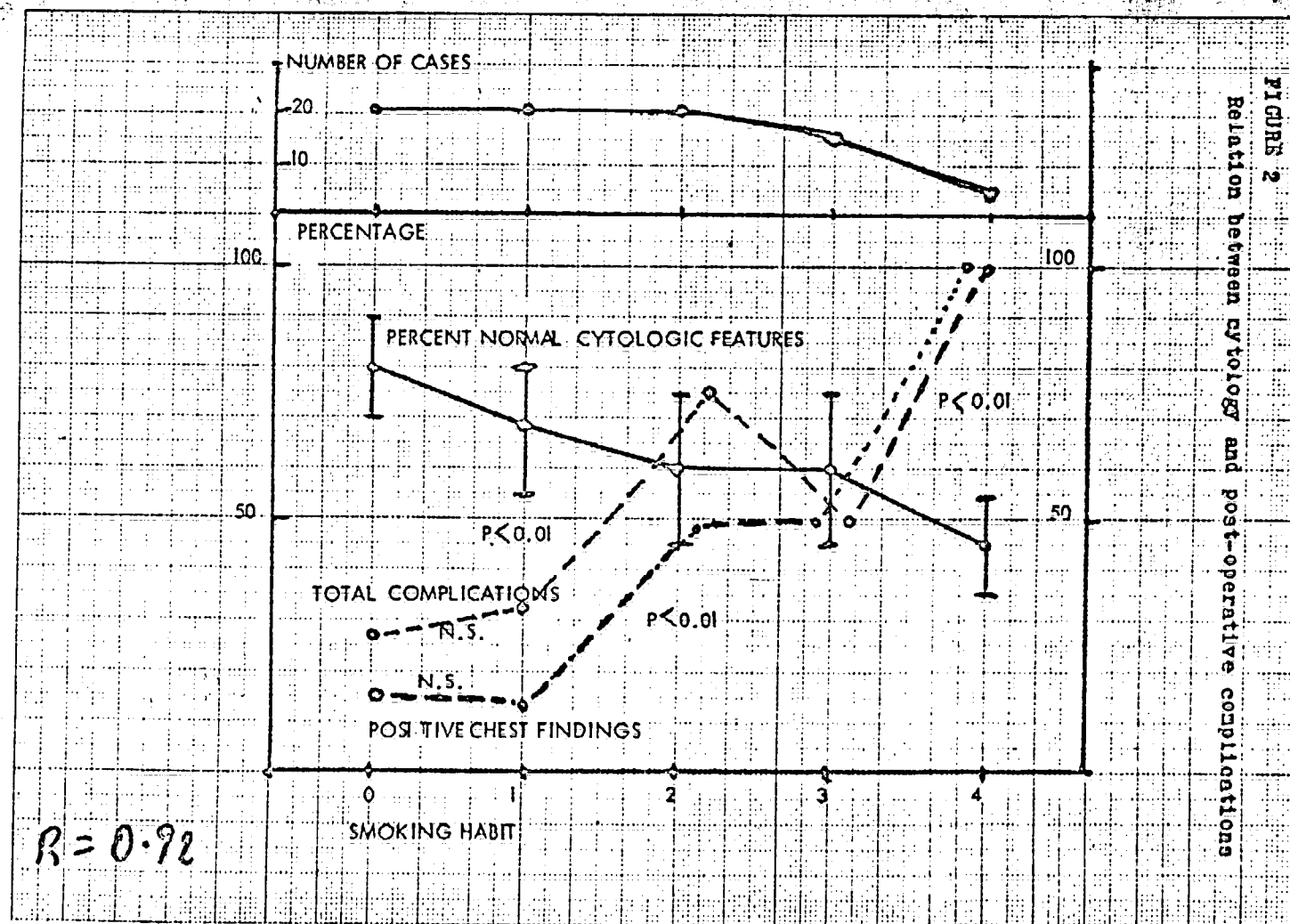
- 1) Chalon, J., Loew D.A.Y., Orkin, L.R. Tracheobronchial cytologic changes during the menstrual cycle. JAMA 218:1928-1931, 1971
- 2) Chalon, J., Loew, D.A.Y., Malebranche, J. Effects of dry anesthetic gases on tracheobronchial ciliated epithelium. Anesthesiology 37:338-343, 1972
- 3) Auerbach, O., Stout, A.P., Hammond, E.C., Garfinkel, L. Changes in bronchial epithelium in relation to sex, age, residence, smoking and pneumonia. New Eng. J. Med. 267:111-119, 1962
- 4) Cigarette smoking and lung ventilation. Comment, British Med. J. of Austr. 2 (11) 68-77, 1964
- 5) Peterson, D.H. et al. Archives of environmental health. 16(2) 215-218, 1968
- 6) Islam, M.S. et al. Pulmonary functions in smokers. Indian J. Physiol. and Pharmacol. 14(31) 165-173, 1970
- 7) Belcher, J.R. World-wide differences in the sex ratio of Bronchogenic carcinoma. Brit. J. Dis. Chest 65:205-221, 1971

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Type of Study	Smoking habit	Rate of decay of cytologic features during anesthesia		
		N	\bar{X}	SD
DRY GASES	Non smokers and light smokers	15	67	15
	Medium and heavy smokers	9	53	9
Cases with 60% Relative Humidity	Non smokers and light smokers	10	85	11
	Medium and heavy smokers	7	85	11

TABLE 1.

Effects of smoking habit on rate of decay of cytologic features during anesthesia in patients breathing dry and 60% humidified gases

Non smokers and light smokers: 0-9 cigarettes a day

Medium and heavy smokers : 10 or more cigarettes a day

N: number of cases, \bar{X} : Mean measurement SD: standard deviation from the mean.

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CARCINOGENESIS AND CHRONIC PULMONARY DISEASES

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC

COMMITTEE:

Dr. Gardner
Dr. Loosli
Dr. Sommers

110 EAST 59TH STREET

NEW YORK, N.Y. 10022

Application For Research Grant

JUL 11 1972

Date: July 5, 1972

History
No. 725C4

725C(Deferred)

725B Act:10/1/71

725A Act:10/1/70

725 Act:10/1/69

1. Name of Investigator(s): (include Title and Degrees)

Principal Investigator: Jack Chalon, M.D.
Assistant Professor, Anesthesiology

Associate Investigator: Dr. Valentin N. Dolorico, M.D., Research Fellow

2. Institution &
Address:

Consultant: Louis R. Orkin, M.D., Professor & Chairman, Anesthesiology
Medical Technician: Miss Cheryl B. Rabinowitz, B.A.

Albert Einstein College of Medicine
1300 Morris Park Avenue
Bronx, New York 10461

3. Short Title of Project:

CHANGES IN TRACHEOBRONCHIAL CYTOLOGY

4. Proposed Starting Date: October 1, 1972

5. Anticipated Duration of this Specific Study: Two years extension

6. Brief Description of Objectives or Specific Aims:

Three years of support by the Council for Tobacco Research, Inc., U.S.A. have allowed us to establish the existence of cytomorphologic and cytochemical changes in the cells of the tracheobronchial epithelium of patients undergoing general endotracheal anesthesia. These include cyclic changes in young women during the menstrual cycle (1) changes due to dryness of anesthetic gases (2,3) and the detection of disturbances in cell anatomy resulting from the inhalation of irritants and the development of malignancies. This study proposes to extend the examination of bronchial washings obtained during anesthesia and include the pre-operative evaluation and post operative follow up of the patient as a whole in order to assess the effect of the cytologic changes already described on the development of physiopathologic changes during the post operative period.

It also proposes to relate to tracheobronchial cytologic response in non-smokers, smokers and previous smokers to the inhalation of dry anesthetic gases and the effect of smoking on the cyclic cytomorphologic and cytochemical changes found in young females during the menstrual cycle

7. Give a Brief Statement of your Working Hypothesis:

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Experimental design and procedures: (Attached separate pages)

8. ~~Details of Experimental Design and Procedures: (Attach Separate Pages)~~

See appendix 2

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

10. Additional Requirements:

A Pulmonary Function Indicator is required
Expendable supplies include

- (1) Glass slides
- (2) Staining materials, fixatives and mounting media
- (3) Photographic film, developing and printing
- (4) Publication expenses (Charts, diagrams, color publications)
- (5) Travel expenses incurred in the presentation of papers and attendance at Medical and Scientific Meetings

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Biographical sketches of all principal and professional personnel (append)

Dr. Jack Chalon - See appendix 3

Dr. Valentin N. Dolorico - See appendix 4

Dr. Louis R. Orkin - See appendix 5

Miss Cheryl B. Rabinowitz, B.A. - See appendix 6

12. List of publications: (Five most recent as pertinent) (append)

See appendix 7

Source: <https://www.industrydocuments.ucsf.edu/docs/gv1000>

13. Budget: (1st year)

A. Salaries (Personnel by names)

Professional

Dr. J. Chalon

Dr. Valentin N. Dolorico

% time

50%

100%

Amount

Full time salaried

Full time salaried

Technical

Miss Cheryl B. Rabinowitz

Fringe benefits 15%

10,894.00

1,634.00

Sub-Total

12,528.00

B. Consumable Supplies (list by categories)

Stains, fixatives, slides,
Photography

1,000.00

250.00

Sub-Total

1,250.00

C. Other Expenses (itemize)

Publications

Travel

700.00

500.00

Sub-Total

1,200.00

D. Permanent Equipment (itemize)

Pulmonary Function Indicator
(Chemitron)

885.00

E. Overhead (15% of A + B + C)

Total

2,247.00

18,110.00

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	13,781.00	1,250.00	1,200.00	none	2,436.00	18,667.00
Year 3						

Signature

Director of Project

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature

Head of Institution

Telephone

Telephone

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Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current

Title of Project	Source	Amount	Duration
Changes in tracheobronchial Cytology	The Council for Tobacco Research, Inc. - U.S.A.	16,192.00	3 years

Pending

1. Humidity and the Patient)
2. Smoking and Chronic Obstructive Lung Disease)

Presently conducted on available fund from the Council for Tobacco Research, U.S.A. - to be expanded if grant is renewed

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APPENDIX I

The Hypothesis envisages the development of routine screening of all patients undergoing general endotracheal anesthesia for the surveillance and study of abnormalities and their possible causes with particular emphasis on bronchogenic carcinoma. It also proposes to scrutinize the effects of dry anesthetic gases in comparison with partially and fully humidified gases and to determine their action on the ciliated columnar epithelial cells of the trachea and bronchi and to evaluate the cytologic data in relation to the pre-intra and post operative follow up of the patients in order to assess optimal desired humidification which will reduce the incidence of post operative complications.

Since we have demonstrated the existence of cyclic cytomorphologic and chemical changes in the tracheobronchial trees of women in the reproductive stage of life and since there is a lower incidence of pulmonary carcinoma in females, some related factor probably protects the cell from inhaled irritants and carcinogens. Figures computed to date indicate that these changes are less marked in female heavy smokers who have a higher incidence of metaplasia from ciliated into goblet cells.

The excellent diagnostic potential and large number of available patients can be correlated to develop guidelines to improved intraoperative care and to interpret some of the abnormalities occurring after exposure to unsatisfactory atmosphere.

Previous history and preoperative evaluation (lung function tests) followed by the intra and post operative follow up of a large number of patients exposed to dry or humidified anesthetic gases may throw some light on the defensive mechanisms of these individuals and their ability to resist the development obstructive lung disease and pulmonary carcinoma.

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APPENDIX 2

A. We have shown that:

Cytologic changes which occur in the endometrium are paralleled by epithelial changes in the tracheobronchial tree. During the past 33 months 2567 patients have been examined and the following methods used.

1. SCREENING: Randomly selected patients from the 10,000 cases undergoing routine general endotracheal anesthesia were examined. As soon as possible after intubation, routine suctioning of the tracheobronchial tree yields sufficient sputum for smearing and immediate fixation. The slides are stained by the Papanicolaou and Periodic Acid Schiff Methods. Mean nuclear position is assessed by examining 300 cells in each slide. If the nucleus is at the base, the middle or the apex of the cell it respectively scores 1, 2 or 3. Thus a total score varying from 300-900 for the 300 cells is possible. Percentages of goblet cells in relation to all columnar cells examined (ciliated plus goblet) are noted. Photography of usual and atypical findings is used for record keeping. All smears are also screened for nuclear and cytoplasmic atypia and are included in the appropriate Papanicolaou Class.

- B. We have also shown (3) that the tracheobronchial ciliated epithelial cells of patients exposed to dry anesthetic gases suffered significant anatomical changes if the time of exposure exceeded one hour.

2. Selected patients undergoing prolonged surgery are studied in subgroups receiving similar introduced relative humidities varying from dry gases to fully humidified gases. All patients are examined pre-

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APPENDIX 2 (CONTINUED)

operatively to assess their pulmonary functions (FEV1, FEV3 and Maximal Expiratory Flow Rate) and relevant past and family histories. They are followed up post operatively and the development of pyrexia, physical chest signs, x-ray abnormalities and \dot{V}/\dot{Q} aberrations noted (from blood gas analysis). Smears taken from these patients at the onset and at the end of anesthesia are examined and damage due to dissection is looked for in the nuclei, cytoplasm, cilia and end plate and assessed numerically by a point scoring system³. The development of cellular changes is correlated with the incidence of post operative complications in the groups receiving dry and humidified anesthetic gases.

Most of the findings of the first 33 months have been published are incorporated in appendix 7. A progress report for the last 12 months will be available at the end of August.

The multiplicity of factors involved demands still larger numbers of patients but the following conclusions can be made:

1. Changes occur in the cells which are definitely related to the menstrual cycle¹
2. Female heavy smokers have less cytomorphologic changes but a greater incidence of metaplasia of ciliated into goblet cells (this series still needs to be expanded).
3. Dry anesthetic gases affect ciliated columnar cells when administered through an endotracheal tube which by passes the moisturizing zone in the nose if anesthesia lasts more than one hour.

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APPENDIX 2 (CONTINUED)

4. Smokers have less normal features in their ciliated epithelial cells than non-smokers but their cells suffer less when exposed to dry anesthetic gases.
5. Patients receiving dry anesthetic gases have an increased rate of post operative complications (23%) than non-smokers. The series studied is still small and further work is needed in that respect.
6. Patients placed on the circle absorber system receive anesthetic gases with varying degrees of humidification. The humidity of these gases can be assessed in relation to fresh gas input, respiratory minute volume and CO_2 production. Nomograms have been drawn by us⁴ to predict the humidity of this system. Because this system generates water vapor which condenses in its valves, bag and tubing, bacterial growth is easily promoted within its midst. We have devised a non-rebreathing humidified system (to be published) in which humidity can be regulated at will and the system kept dry and sterile.
7. Abnormal cells (so far atypia falling into class III) appear from time to time in smears.

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REFERENCES

ALL DOCUMENTS INCLUDED IN APPENDIX 7

1. Chalon J, Loew DAY, Orkin LR. Tracheobronchial Cytologic changes during the Menstrual Cycle. JAMA 218:1928-1931, 1971
2. Loew DAY, Klein SR, Chalon J. Volume-controlled relative humidity using a constant-temperature water vaporizer. Anesthesiology 36: 181-184, 1972
3. Chalon J, Loew DAY, Malebranche J. Effect of dry anesthetic gases on tracheobronchial ciliated epithelium. Anesthesiology (in press)
4. Chalon J, Kao ZL, Dolorico VN, Atkin DH. Humidity output of the circle absorber system. Submitted to Anesthesiology

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 6, 1973

Grant Application No. 766A

To: The committee comprising Drs. Andervont, Gardner, and Huebner

Subject: Richard A. Lerner, M.D., Scripps Clinic and Research Foundation,
LaJolla, California
Continuation application No. 766A
"Studies on Persistent Viral Infection"

History

The grant now current, \$49,495, supports the last year of a three year program.

The pending application, in the amount of \$64,385, requests "continuation", and hence has no priority in competition.

Documents Submitted (attached)

Application dated January 26, 1973 (25 pages plus biographies and bibliographies). Included is a summary progress report on pages 5 and 6.

F.W.N.
F.W.N.

FWN:wg
Encls.

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THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application For Renewal of Research Grant

(Use extra pages as needed)

First Renewal ☒

Second Renewal ☐

Date: 1/26/73

1. Principal Investigator (give title and degrees):

Richard A. Lerner, M.D., Associate Member

2. Institution & address:

Scripps Clinic and Research Foundation
476 Prospect Street
La Jolla, California 92037

3. Department(s) where research will be done or collaboration provided:

Department of Experimental Pathology

4. Short title of study:

Studies on Persistent Viral Infection

5. Proposed renewal date: July 1, 1973

6. How results to date have changed earlier specific research aims:

Some significant observations have been made on continuous lymphocytes established from the New Zealand mice and a unique C-type virus has been isolated. As will be described in detail below, we intend to add to efforts already in progress a number of studies based on this new observation.

7. How results to date have changed earlier working hypothesis:

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8. Any additional facilities now required? Describe briefly:

As described in detail in the Methods section, we intend to do a large number of studies to determine the number of gene copies of the new virus which we have isolated in mice. Also we intend to determine the evolutionary history of this malignant oncogene. All the studies, and those regarding evolutionary conditions in particular, necessitate extreme precision in determining the melting kinetics of the nucleic acid hybrids. The Acta 5 recording spectrophotometer which we are requesting will enable us to study these parameters. As you will see from the budget, we have not asked for a substantial increase in funds, but rather are asking for this particular piece of equipment and have deleted some other items in order to be able to purchase it. Also, we have now received some funds from the National Science Foundation; however, they are not to be used for the work proposed in this request and at any rate they would not be sufficient to enable us to carry out this problem because of its increased scope. The increase in direct costs is approximately \$8,000 to accommodate a two-fold increase in professional staff since our last report.

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

As will be described below, our group has increased substantially in size. Two notable additions to the staff are David Kohne and Fred Jensen. We feel that these additions greatly increase our depth in tissue culture and nucleic acid chemistry. The curriculum vitae of these two investigators are appended.

10. Append outline of experimental protocol for ensuing year.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

See attached list.

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12. Summary progress report (append in standard form or separate document, unless recently submitted)

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

	% time	Amount
Richard A. Lerner	50%	-0-
Frank J. Dixon, Consultant	--	-0-
David Kohne	100%	-0-
Fred Jensen	100%	-0-
Alvaro Puga	100%	9,527
Stephen J. Kennel	50%	-0-
Bert C. Del Villano	100%	-0-

Technical

Catherine Morris, Technician	100%	8,618
Part-time services of: secretary, animal caretakers, photographer, glass washers, histology technician, EM technician, machinist & electronic repairman		6,967

Sub-Total for A 25,112

B. Consumable supplies (by major categories)

Tissue culture media, glassware and plasticware	6,000
Isotope labeled compounds	3,000
Liquid scintillators and vials	3,000

Sub-Total for B 12,000

C. Other expenses (itemize)

Photography, histology, part-time use of electron microscope	4,092
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Sub-Total for C 4,092Running Total of A + B + C 41,204

D. Permanent equipment (itemize)

Beckman Acta 5 spectrophotometer for work in nucleic acid chemistry	17,000
--	--------

Sub-Total for D 17,000

E. Indirect costs (15% of A+B+C)

E 6,181Total request 64,385

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14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Studies on persistent viral infection	Council for Tobacco Research #766	\$49,495	7/1/72-6/30/73
Molecular structure of the immunoglobulin receptor in continuous cultures of diploid human lymphocytes	National Science Foundation GB 34296	\$65,000	6/1/72-11/30/74
Immunopathology of virus infection (Salary - RAL)	NIH Career Development Award AI-46372	approx. \$125,000	7/1/70-6/30/75

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Richard A. Lerner, M.D.Signature Richard A. Lerner Date 1/29/73Telephone 714/459-2390, ext. 470
Area Code Number Extension

Responsible officer of institution

Typed Name Edmund L. Keeney, M.D.Title President and DirectorSignature Edmund L. Keeney Date 1/30/73Telephone 714/459-2390, ext. 207
Area Code Number Extension

Checks payable to

Scripps Clinic and Research Foundation

Mailing address for checks:

Mr. O.K. Kincaid, ControllerScripps Clinic & Res. Fndn., 476 Prospect St.,
La Jolla, Calif. 92037

1003538404

10. Experimental Protocol

A. Introduction

During the first three years of our research program under the auspices of the Council for Tobacco Research-U.S.A., Inc., accomplishments in a number of areas have been made.

First, we decided that since the problems pertinent to the biology of cancer are broad in scope it would be necessary to form a group of investigators with multiple talents to approach the problem. This has now been accomplished and we have a group consisting of individuals with expertise in cell biology, virology, immunology, pathology, medicine, nucleic acid and protein chemistry (curriculum vitae appended). The collaborative efforts of this group have proven effective since a number of significant gains have been made:

1) A new species of DNA associated with the plasma membrane of lymphocytes has been described and our results have now been confirmed in a number of laboratories.

2) Methods for radioiodination and isolation of polypeptides from the surface of cells have been established. Currently, any plasma membrane associated polypeptide against which one has an antisera can be isolated (see below).

3) 25 different continuously growing lymphocytic cell lines have been established from the NZB, NZW and (NZB x NZW) F_1 hybrid mice. The cell lines were shown to have a marker chromosome and a deletion involving the X chromosome. A new "C type" RNA containing tumor virus was isolated from these cell lines. This virus can be obtained in large amounts, contains reverse transcriptase, and has already been supplied to a number of investigators interested in cancer biology. Of considerable interest is the fact that when 1.0×10^6 syncytial forming units of this virus were injected into newborn (NZB x BALB/c6) F_1 and (BALB/c6 x NZB) F_1 mice they developed both leukemia and lupus erythematosus (see below).

4) Methodology for immunologic study of the fates of nuclear and nucleolar macromolecules has been described. These studies can now be extended (see below).

5) Infection of human lymphoblasts with RD114 virus was accomplished and an interesting biological effect was observed.

In collaboration with Drs. Berge Hampar and Bob McAllister, a series of experiments were undertaken to determine if four different continuous lymphocyte cell lines could be infected with RD114 virus. These experiments were undertaken for two reasons:

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i) It would be technically advantageous if suspension cultures which produce RD114 virus could be obtained

ii) We were interested in the morphological and biochemical effects of this virus on diploid human cells.

Several interesting results were obtained.

First, when the cells and/or culture fluids were assayed by electron microscopy, RNA dependent DNA polymerase, gs antigens, or focus formation, it was clear that they were infected and producing RD114 virus. Thus, the technical objectives of the research protocol were realized. More interesting, however, was the observed alteration in the cell growth pattern and morphology after infection. The cells first formed clumps, then large giant cells with an increase in the ratio of nuclear/cytoplasmic material. This "CPE" in our experience is unique to RD114 virus and has not been seen with VSV, polio, Arbo, Kirsten-Moloney or myxoviruses. The process induced seems to be either syncytial formation and/or endoreduplication of the nucleus but further studies will be necessary to decide between these alternatives. Autoradiographic studies and karyotypes are being done. If it can be confirmed that the process involves endoreduplication of the nucleus then further studies on the molecular mechanisms by which a RNA tumor virus causes a derangement in the control of the "balance" between nucleic acid replication and cytokinesis will be carried out.

An important aspect of these findings is that they suggest, as Hampar has said in his memo of 12/7/72, that "a number of parameters be checked before any attempt is made to inoculate inactivated virus vaccines". This is especially important since exposure of lymphoid cells to RD114 virus presumably inactivated with β -propiolactone leads to rapid appearance of syncytia. Furthermore, after 3 weeks, even using this "inactivated" virus there was evidence that virus replication was occurring.

Our methods for a continuing study of most of the problems outlined above have been detailed in previous reports. In this report we will concentrate on some new approaches which based on our experience of the last three years appear to be technically feasible and of conceptual importance.

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B. Detailed Description and Rationale for Our Additional Research Effort

In addition to continuation of the work that has already been in progress over the last 3 years, we have undertaken a new effort. The conceptual aspects upon which this new approach is based and the methodology with which to study the problem have been developed in our laboratory during the first 3 years of this program.

It is well known that a tumor evokes a cellular and humoral immune response in its host. Until recently it was assumed that the immune response was protective and continued growth of the neoplasm was due to the fact that the immune defenses were overcome by the tumor. However, we now know that antibodies formed in the process of combating the tumor can actually be injurious to the host. The most obvious effect is the direct consequence of synthesis of "enhancing antibodies". These antibodies have been well studied by the Hellstroms and others and are thought to combine with tumor specific transplantation antigens (TSTA) on cell surfaces and in some way interfere with the potentially protective effect of a second cytolytic antibody. A second way in which the immune system may be involved in carcinogenesis is by activation of a latent viral genome. The work of Schwartz, Hirsch and Cornelius has shown that graft vs. host reactions can induce lymphoid tumors in suitable strains of mice. The tumor is of host origin and presumably caused by activation of a latent viral genome because, as Hirsch and Schwartz have shown, the graft vs. host reaction is known to activate an RNA containing tumor virus. A phenomenon which has not been studied as well as those mentioned above is the generation of antibodies and/or phlogogenic antigen-antibody complexes in patients or experimental animals with tumors. These antibodies and/or immune complexes are formed because during carcinogenesis a number of immunogens such as viral polypeptides, nucleic acids, "neo-antigens" on cell surfaces, etc. are generated. A study of the immune response to these immunogens is of extreme importance for two main reasons. First, antibodies and/or antigen-antibody complexes may have an injurious effect on the host. Second, as a research tool antibodies may have predicative value since once the nature of the antigen against which they are directed is known one has some insight into what process the host is attempting to prevent. For example, one of the best places where one might search for candidate human tumor virus is in patients who do not have tumors but in whom there is immunologic evidence that replication of a tumor virus is occurring. Recently, we tested the validity of these concepts in a preliminary study of the New Zealand mice. Since the results of our studies form the basis for this grant request, they are outlined here. The NZB and (NZB x NZW)_F₁ mice have a disease which resembles human systemic lupus erythematosus. During the course of their disease these mice make a plethora of antibodies including those to:

- dS DNA
- SS DNA
- dS RNA
- SS RNA
- nucleolar RNA
- DNA-histone complexes
- reverse transcriptase
- GSA antigen
- gs antigen

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In spite of this overwhelming evidence that the host is synthesizing antibody to virtually every structural polypeptide, enzyme, TSTA antigen, or nucleic acid intermediate which an RNA tumor virus might code for or induce during its maturation, these mice seldom get tumors. This, therefore, seemed an ideal model system in which to test our concept that "immunologic tracks" can give clues as to where one can search for oncogenic viruses even and perhaps particularly where the host does not have a tumor. Accordingly, 25 continuous lymphocyte lines were established from NZB, NZW and (NZB x NZW)_{F1} mice. Once established, all the lines began to synthesize a virus and had easily demonstrable neo-antigens on their surface. The virus had a C-type morphology, a density of $1.16 \text{ gm} \times \text{cm}^{-3}$, contained 70S RNA and RNA dependent DNA polymerase and has been designated as the Scripps leukemia virus 60A (SLV 60A). An additional finding of interest was that all cell lines studied had a marker and a deleted X chromosome. Embryos from New Zealand mice were shown to be mosaic for the chromosome marker. It is the purpose of our present effort, for which these funds are requested, to extend these findings in both murine and human systems. Obviously, the interaction between this virus and its host is a complex process and studies ranging from the evolution of this gene in murine genomes to the nature of the immunogens induced by infection are pertinent. Therefore, we have put together a group with expertise in virology, tissue culture, cell biology, immunology, pathology and protein and nucleic acid chemistry to approach the multiple aspects of this problem. The collaboration of this staff is proving effective as evidenced by the progress to date and is the basis of our request for continued support of a broadly based approach to the study of viral oncology with special emphasis on immunoprophylaxis and immunotherapy.

I. The immunopathology of injection of mice with SLV virus

In a preliminary study, (BALB/c x NZB)_{F1} and (NZB x BALB/c)_{F1} newborn mice were injected with 10^6 syncytial forming units (SFU) of SLV 60A virus. Within 3 months, one half of the mice had developed leukemias and/or lymphomas with generalized involvement of lymphoid tissues and parenchymatous organs. In addition, all animals injected with virus developed strong antinuclear antibodies by two months of age. 3/20 injected mice had glomerular lesions resembling immune complex glomerulonephritis by 3 months.

Thus it appeared that the purified virus preparation might be responsible for both leukemia and autoimmunity. The proposed investigations are designed to further investigate this phenomenon.

(a) Effect of SLV on a variety of murine strains

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Since both the incidence of spontaneous leukemia and autoimmunity are strain dependent in mice, we propose to study a variety of murine strains to determine the incidence of synthesis of anti-DNA antibodies and occurrence of leukemia after injection with SLV virus.

Preparation of virus. Virus will be prepared by isopycnic centrifugation to density of 1.16 gm in the CF-35 zonal rotor. In our hands, approximately 5 liters/hour of supernatant fluid can be cleared in this fashion with a virus yield of approximately 1 mg of virus/liter. Following this, the virus is sedimented to its isopycnic density in a 14.5 to 46.0% sucrose gradient (w/w) at 27,000 rpm for 4.5 hours in the SW27 rotor. Virus prepared in this fashion is then titrated for infectivity by syncytial formation in the XC assay and for net yield by determination of the amount of gs-1 antigen present. The XC assay used is that of

Klement, Rowe, Hartley and Pugh, and the gs-1 protein is determined by a quantitative radioimmune assay developed in this laboratory which is being used routinely and will form the basis for a number of the quantitative studies described below.

(b) Effect of 10^6 syncytial forming units of SLV virus in a variety of murine strains

The strains of mice to be studied were selected to include those with both high and low incidences of leukemia and/or autoimmunity. 8 liters of the following murine strains will be used: NZB x BALB/c6, NZB, NZW, (NZB x NZW) F_1 , NZW x BALB/c6, C57Bl/6, C57 Brown 10, SWR/J, BALB/c, AJAX. 5 liters from each strain will be injected with 10^6 syncytia forming units of SLV and 3 liters will serve as controls. At 30 day intervals each mouse will be studied for the synthesis of antinuclear antibodies and for proteinuria. When evidence of disease is present the mouse will be bled and a complete autopsy performed. The serum will be studied for the presence of antinuclear antibody and for quantitative determination of the amount of anti dS DNA and anti SS DNA. The methods for determination of antinuclear antibody and quantitation of the amount of anti dS DNA and SS DNA present in the serum are well established and in routine use in this laboratory. White blood cell and red blood cell counts and a differential will be done on all blood samples. In addition to routine sections for pathologic diagnosis, portions of the kidney will be snap frozen for immunofluorescence to determine if IgG, complement, nucleic acids, and gs-1 protein is deposited in the form of immune complexes in the glomeruli. The methodology for a proper immunofluorescent study has been worked out over the last 10 years in this laboratory and adequate facilities as well as appropriate antisera are available. Also, samples of tumor and kidney will be studied by electron microscopy.

(c) Determination of the relationship of the dose of SLV virus to the onset of leukemia and autoimmune disease in the (BALB/c x NZB) F_1 hybrid mouse

Since we already know that when this strain of mice is injected with 10^6 syncytial forming units of SLV virus they develop leukemia and synthesize antinuclear antibody, a more detailed study to determine if a temporal separation in the onset of leukemia and autoimmunity can be achieved.

35 (BALB/c x NZB) F_1 liters will be injected with either 10^6 , 10^4 , 10^2 , 10^1 or 10^0 syncytial forming units of SLV virus. Each mouse will be studied as described above.

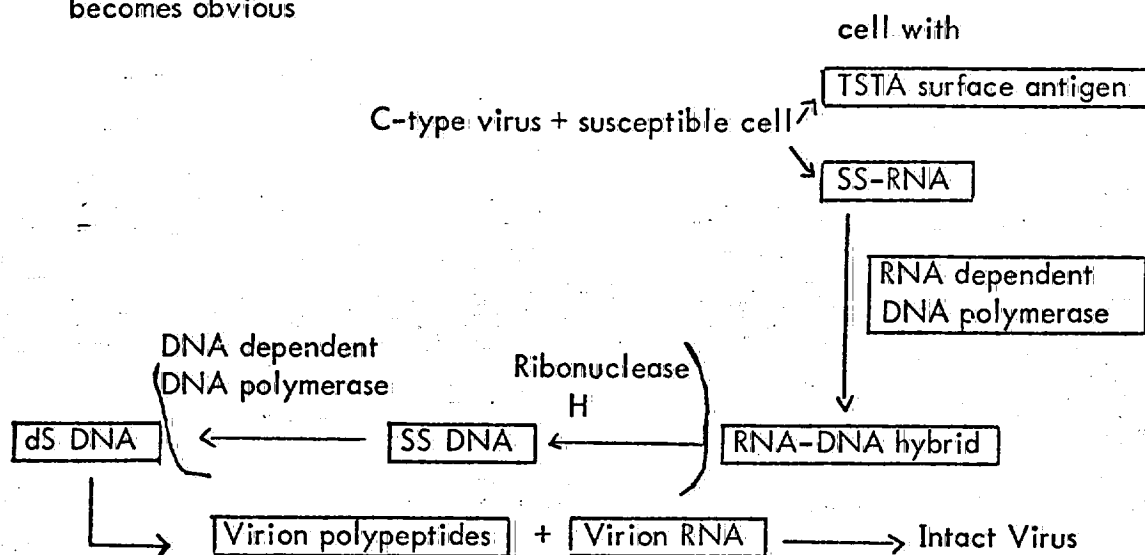
We expect to learn from the data obtained from these experiments the best combination of viral dose and murine strain necessary to induce both autoimmunity and leukemia. It is further anticipated that by appropriate combinations one may be able to induce autoimmunity without leukemia or vice versa.

II. Relationship of autoimmunity to the amount of gs-1 antigen present in the serum and spleen of ageing New Zealand mice

One of the premises upon which this request is based is that the onset of autoimmunity in the New Zealand mice is related to the replication of RNA tumor virus since during replication this virus presents a genetically susceptible host with a great number of potential immunogenic macromolecules. If we review how the virus replicates, the nature of the immunogenic load

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becomes obvious



The immunogens against which the NZB or the (NZB x NZW) F_1 are thought to make antibodies are enclosed in a box . Of course, in the case of the antibodies against the nucleic acids it is not certain if their foreign nature terminates tolerance to autologous nucleic acids or if they are a specific immunogen.

Regardless, since the autoimmune disease in the (NZB x NZW) F_1 progresses with age, eventually leading to death, it is important to quantitate the amount of virus and/or virion polypeptides synthesized at different ages and correlate this with chemical and pathologic symptoms of autoimmunity.

Methods for determining the amount of antibody against SS DNA or dS DNA in the serum of mice is a technique already worked out and in use in this as well as other laboratories. Also the methodology to make the immunopathologic diagnosis of autoimmunity has been well established by us and other investigators.

Recently we have established methodology for quantitation of the gs-1 antigen in serum and organ extracts. A great deal of effort has been put into development of a radio-immune assay which is sensitive, accurate, highly quantitative, and sufficiently automated so that a large number of samples can be studied. The details of this method are outlined below:

gs Radioimmune Assay

I. Antigens

1. Use purified gs-1. Use IgG from the species in which the anti gs was raised.
2. Iodinate gs with I^{125} to achieve approximately 10,000 cpm per 0.5 ng gs protein (50 μ g gs P + 5 mc I^{125} + 5 μ g chloramine T - 5 min. + 5 μ g $Na_2S_2O_5$)
Iodinate IgG with I^{131} to achieve approximately 10,000 cpm per 50 ng IgG protein. (2 mg IgG P + 4 mc I^{131} + 25 μ g chloramine T - 10 min. + 25 μ g $Na_2S_2O_5$)

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Post-iodination $>95\%$ I^* is protein bound (as determined by precipitation with 10% TCA).

3. Prepare stock solutions of I^* proteins in BSA (2 mg P/ml in 0.1 M borate buffer, pH 8.3 to 8.5). Stock gs = 1 μ g P/ml and stock IgG = 10 μ g P/ml. Freeze stock solutions at -20°C in aliquots appropriate for daily use.
4. Prepare working antigen in BSA (2 mg P/ml in 0.1 M borate buffer) so that gs is at 10 ng/ml and IgG is at 1 μ g P/ml (1-10 dilutions of stocks). Add 1 μ c Na²² per ml. 50 μ l per test then contains 0.5 ng gs protein and 50 ng IgG protein.

II. Antibody

1. Antibody should be hyperimmune to gs but not necessarily specific for gs, if a pure gs antigen is available for iodination (i.e., antibody to fetal calf serum will not interfere in the quantitation of gs in this assay). We are currently using a guinea pig anti MuLV gs obtained from Dr. Gilden.
2. Dilutions of antibody are made in de complemented normal rabbit serum, diluted 1-10 in 0.1 M borate buffer.

III. Ammonium Sulfate

Optimum $(\text{NH}_4)_2\text{SO}_4$ concentration for precipitation of I^{125} gs bound to antibody is dependent on the species in which the hyperimmune anti gs is prepared. With the guinea pig system we are currently using a final concentration of 42.5% ammonium sulfate (4°C) is optimal.

IV. Anti gs dilution for inhibition assay

Since this system is based on the competition of I^{125} gs with cold unknown gs for available antibody sites, it is necessary to carry out the assay in excess I^{125} gs (i.e., in excess antibody small amounts of cold gs would not be detectable). We have chosen as our base line that dilution of antibody which is capable of binding 50% of the I^{125} gs added.

1. Prepare guinea pig anti gs dilutions in 10% Δ NRS, starting with a 1-50 dilution and serial 1-5 dilutions thereafter (i.e., 1-50, 1-250, 1-1250, 1-6250 and 1-31,250)
2. In triplicate add 50 μ l diluted antibody plus 50 μ l 10% Δ NRS + 50 μ l working I^* antigen in Beckman microfuge tube (400 μ l)
3. Mix and incubate 30 minutes at 37°C and one hour at 4°C
4. Add 100 μ l saturated (at room temperature) ammonium sulfate which equals a final concentration of 42.5% ammonium sulfate at 4°C .
5. Mix well and incubate at 4°C for 30 minutes
6. Centrifuge in Beckman microfuge for 5 minutes
7. Aspirate approximately 90% of supernatant, cap and count in Nuclear Chicago automatic gamma counter that is set to record I^{125} , I^{131} and Na²² counts.
8. The Na²² acts as a volume marker and the I^{131} IgG precipitin acts as a monitor of the technique. The I^{125} specifically bound can be determined. We have programmed a Wang computer to do this calculation directly from the Nuclear Chicago tape read out.
9. On semi-logarithmic paper plot % gs bound on the linear scale versus anti gs dilution on the logarithmic scale. Determine at what antibody dilution 50% of the I^{125} gs is bound.

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V. Control Inhibition Curves

In order to equate the inhibition achieved by unknown materials suspected of containing gs, it is necessary to determine what inhibition is achieved by inhibitors of known concentration. We use a NZB gs obtained from Dr. Gilden that contains 30 µg gs/ml.

Procedure (in triplicate in 400 µl Beckman microfuge tubes)

1. Base line control = no inhibition
50 µl 10% ▲NRS + 50 µl diluted guinea pig anti gs + 50 µl I* Ag
2. Inhibitors (diluted in 10% ▲NRS)
50 µl inhibitor of known conc. + 50 µl ab + 50 µl I* ag
3. Mix tubes and incubate at 37°C for 30 min. and at 4°C for one hour
4. Add 100 µl saturated (at room temperature) ammonium sulfate
5. Mix well and incubate at 4°C for 30 min.
6. Centrifuge 5 minutes in Beckman microfuge
7. Aspirate 90% of supernatant, count and determine % I¹²⁵ gs specifically bound
8. Determine % inhibition of each known inhibitor by comparison with base line control

$$\text{i.e., \% inhibition} = \frac{\% \text{ bound base line control} - \% \text{ bound exp}}{\% \text{ bound base line control}} \times 100$$

9. On semi-logarithmic paper plot % inhibition on the linear scale versus added gs inhibitor (ng P/ml). Any inhibition values achieved with unknown substances (i.e., sera, tissue extracts, etc.) can now be quantitated from this standard inhibition curve.

We have already shown that the assay detailed above can be utilized to quantitate the amount of gs-1 antigen in the serum and/or organ extracts of mice. We now propose to bleed NZB, NZW, (NZB x NZW)F₁ serially and determine the amount of gs-1 antigen present in their serum. The same animals will be studied for the development of autoimmune disease. It is anticipated that the amount of gs-1 antigen present in the serum will be a "marker" of the degree of autoimmunity. If this is so, the concept that replication of the virus parallels the development of autoimmune disease will be strengthened.

III. Isolation of virus induced cell surface associated polypeptides from continuously growing New Zealand lymphocytes

One of the antigens against which the New Zealand mice presumably make antibody is a virus induced cell surface polypeptide which in the past has been called the GSA antigen. It is important to know the nature of this antigen because it may be one of the immunogens involved in immune complex formation and might also be a primary target of the host's immune defenses. We were able to show by immunofluorescent studies that it was present on the surface of all the lymphoid lines established from the NZW, NZB, and (NZB x NZW)F₁ hybrid mice. Since these cells can be grown to large numbers and can be harvested as uniform suspensions without the use of proteolytic enzymes, the system is ideal for the isolation of virus cell surface polypeptides. Accordingly, we approached the problem utilizing methodology which has been established over the past two years in this laboratory for radioiodination and isolation of cell surface polypeptides. In a preliminary study, cell surface polypeptides of

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SCRF 60A cells were labeled with ^{125}I by the lactoperoxidase method. Several criteria were considered in setting conditions for effective labeling of these and other cells: 1) constant protein derivatization, 2) maximum iodine incorporation and 3) suitable cell viability. To satisfy the first criterion, we used a constant amount of cold carrier I^- (10^{-5} M). This practice allows a variable incorporation of radioactive iodide (negligible concentration) without changing the number of iodides incorporated per protein molecule. This carrier concentration also allows the enzyme to work in an efficient substrate concentration range. We found that the concentration of H_2O_2 used in the reaction is very important in achieving a large iodide incorporation without oxidizing vital cell components. The optimum concentration varies with the amount of enzyme and substrate in the system. For this reason, standard conditions of 2×10^7 cells/ml, 10^{-5} M KI and $3.3 \times 10^{-7}\text{ M}$ lactoperoxidase (Phillips & Morrison, 1970) were adopted. With these set conditions, the effects of H_2O_2 concentration on labeling and cell viability were studied. Maximum incorporation was achieved at $44\text{ }\mu\text{M}$ H_2O_2 with cell viabilities of 90%. This viability increases to 99% when cell washes are done using cold Earle's salt solution rather than room temperature phosphate buffered saline. Multiple additions of this concentration of H_2O_2 did not result in linear increments of I^- incorporation demonstrating that H_2O_2 is not in limiting concentration. Using these conditions, the reaction is over in less than one minute. This rapid labeling allows fast processing and therefore minimal cell exposure to the relatively harsh labeling conditions. Following labeling, the infected cells were disrupted with NP40 and the labeled polypeptides precipitated by the "sandwich" technique. The first antisera was obtained from rats bearing syngeneic Moloney virus induced tumors. Five different batches of this antisera were used. After incubation of the labeled polypeptides with the first antisera for 30 minutes, an appropriate amount of rabbit anti rat IgG was added to precipitate all the rat IgG. Freeze dried immune precipitates were solubilized in $100\text{ }\mu\text{l}$ of 1% SDS containing 8 M urea at 60°C for 1 hour. 1-2% mercaptoethanol was added where reduction of disulfide bonds was required. The sample was layered on the top of $6 \times 100\text{ mm}$ 6% SDS polyacrylamide gels and electrophoresis was carried out at 15 ma/gel for 2 hours. Marker proteins labeled with ^{131}I were included in every gel. One mm fractions were sectioned from frozen gels using a Joyce-Lobel automatic gel slicer, counted, and the data corrected for crossover efficiency. Control studies utilizing normal rat sera and a nonspecific immune reaction ("trapping control") were carried out. We were able to reduce nonspecific trapping to about 1% of the labeled protein solution. In addition to radioiodination, cells were labeled with ^3H -glucosamine and processed as described above.

In every experiment, a single polypeptide with a molecular size of approximately 60,000 daltons was isolated. From the experiments with ^3H -glucosamine we were able to show that the polypeptide we have isolated is a glycopeptide. Since this polypeptide only combines with sera from tumor bearing rats and appears only on the surface of cells transformed with RNA tumor virus, it can be classified in the broad group of TSTA antigens. Of course, it is not yet known if this polypeptide is a primary gene product or is coded for by the host genome. If the latter is true or if there is substantial gene relatedness among the RNA tumor viruses, this glycopeptide may be a general marker of transformation of cells with RNA tumor viruses.

We propose to study, as described above, the cell surfaces of cells infected with Moloney, Gross, Friend, RD114 and Rauscher virus with a single antisera pool to determine if a similar polypeptide is on the surface of all these cells. From these studies we expect to be able to determine if the polypeptide we have isolated is a general marker of transformation with RNA tumor viruses and if it is present in species other than murine.

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Also, we would like to know when the New Zealand mice make antibody against this polypeptide. Accordingly, sera from NZB, NZW and (NZB x NZW) F_1 hybrid mice of different ages will be utilized as the first antisera (in the sandwich technique, see above) to isolate the cell surface glycopeptide (TSTA) from continuous lymphocyte lines transformed with SLV 60A virus. In every case the glycopeptide will be studied by gel electrophoresis as described above. From these studies we expect to be able to determine the taxonomic classification of this glycopeptide and how the appearance of antibodies to it in the New Zealand mice correlates with the development of autoimmunity.

IV. Studies on the Structure of SLV Virus

Since NZB and (NZB x NZW) F_1 mice make antibodies to a number of structural virion polypeptides of the murine RNA tumor viruses, it is of considerable importance to characterize the structural polypeptides of SLV virus and determine exactly which of these the New Zealand mice make antibodies against. Two different methods for labeling of the virion polypeptides with isotopes will be utilized. The lactoperoxidase method of iodination modified by attaching the lactoperoxidase to sepharose 4-B beads was found to be effective in labeling intact virions. The large size of the sepharose 4-B beads helps insure that only the polypeptides on the virion surface are iodinated. In addition, all the virion polypeptides will be uniformly labeled with mixed ^{14}C amino acids. Following these two quite different methods of labeling, the virions will be disrupted with SDS. The solubilized proteins will be reduced with mercaptoethanol and characterized by polyacrylamide gel electrophoresis as described above.

In some experiments specific polypeptides labeled by one or the other method will be precipitated by selected antibodies utilizing the sandwich method (see above) (e.g., against gs-1, reverse transcriptase, neutralizing) and then the precipitate will be dissolved and studied by acrylamide gel electrophoresis (see above). From these studies we expect to not only be able to characterize the nature of the virion polypeptides but also, because of the use of specific antibodies, to put individual polypeptides into the proper biological perspective. For example, those polypeptides associated with the virion surface which react with neutralizing antibody are most likely to be important in the host defense against replicating virus.

V. Taxonomy of SLV 60A Virus

It is important to determine the relationship of the SLV virus to other murine RNA tumor viruses. Accordingly, the standard reciprocal neutralization assay will be carried out. The necessary cell lines, viruses and antibodies to carry this out have already been supplied by the NCI.

Of course, because of the "third position effect" in the genetic code, any type of determination of the polypeptide structure does not necessarily confirm a genotypic relationship. Accordingly, the degree of "relatedness" of the SLV 60A genome to other murine genomes will be determined as described below. When these studies are completed, we expect to understand the exact relationship of the SLV 60A virus to other murine RNA containing tumor viruses.

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VI. Evolution of the SLV 60A gene and genotypic relatedness to other murine RNA tumor virus genomes

Since the development of autoimmunity is highly strain specific, it is important to understand how these genes evolved, how many copies are present in the genome of the NZB, (NZB x NZW)F₁ hybrid, and the NZB mice, and the genotypic relatedness of this genome to other RNA tumor viruses. The experimental rationale for these studies is described below.

However, since many of these studies depend on preparing a DNA transcript of the RNA genome of high specific activity to be used as the "probe" to carry out genetic studies, a preliminary study of the feasibility of this using banded SLV 60A virus was carried out.

The virus was found to have a highly active RNA-directed DNA polymerase which readily copies the endogenous template. The rate of incorporation of ³H-TTP is linear for approximately 2 hours. The DNA product has a specific activity of approximately 3.0×10^7 DPM/ μ g of DNA, and as has been reported, in the presence of actinomycin D, remains associated with 70S RNA. The T_m of the RNA-DNA hybrid product is approximately 77.5°C in 0.14 M $PO_4^{=}$ as studied by release from hydroxyappetite. This T_m is correct for well matched DNA-RNA hybrids with G+C contents of approximately 40% by the hydroxyappetite method. For example, this is the approximate T_m for ribosomal genes complexed to their RNA gene product when studied by this method. Finally, the recovered product could be shown to reanneal quantitatively with purified 70S viral RNA. Recently it has been reported that the SS DNA transcript is representative of the entire genome and thus a suitable probe for determining gene "copiedness" and genotypic homology among viruses.

Cells cultured from NZB mice produce C-type viruses in large quantity. Many different cell lines from these mice have been established and all of these lines produce C-type viruses in culture. These lines provide a unique opportunity to study many different aspects of the interaction between cell and virus. We plan to study the nucleic acids of these viruses and cells in order to ask some basic questions about the nature of the virus itself, the evolutionary history of these viruses and the virus interaction with cells.

The research proposal here will revolve around 3 separate but related questions: (A) How many different C-type viruses are produced by these cells? (B) What is the evolutionary history of the C-type viruses produced by these cells? (C) How many NZB C-type virus genomes are present in each of the different cell lines and in normal cells from various mouse strains?

Part A may provide us with some idea as to the potential number of mouse C-type viruses which are present in a mouse cell. This has great implications regarding the probability of a cell expressing a virally related tumor or disease. Part B will hopefully provide us with information concerning the origin of viruses. It should also provide a framework which will enable us to judge whether a particular virus is in fact a mouse virus. Part C is important for the interpretation of data obtained in A and B and may also provide us with an indication of the types of molecular interactions which can occur when cells and viruses interact.

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A. How many different C-type viruses are present in these cells? What is the relationship of the NZB virus RNA to other C-type mouse viruses?

It is not as yet clear whether different C-type viruses are being expressed in each of the different NZB cell lines. This question can be approached in at least two different ways, immunologically or by nucleic acid hybridization techniques. In the overall effort of this study both techniques will be tried. The nucleic acid hybridization technique will be more sensitive at detecting differences between the virus. It is possible that two viruses could have exactly the same protein complements and still be different at the level of their nucleic acid sequence. This situation might arise, for example, if at some time during evolution of the mouse several copies of a particular C-type virus genome were integrated into the mouse DNA. Thus, each mouse cell would have several copies of the viral genome. Section C will discuss the determination of the "copiedness" of a viral genome in a cell. Each of these multiple genomes could then diverge from one another with time and at some later time the genomes would be similar but not identical to one another. With enough time and change, a new virus might be formed. The repeated DNA sequence present in all mammals goes through an evolutionary history similar to this.

Two viruses can have exactly the same proteins and different nucleic acid sequences because of a characteristic of the genetic code called synonymous codons. It is known that a sequence of 3 nucleotides codes for an amino acid. The rules of the genetic code are flexible enough so that many amino acids can be coded for by more than one triplet sequence of nucleotides. For example, the amino acid leucine is coded for by the triplets, CUU, CUC, CUA, CUE. It is clear that a mutation could occur in the third position of any one of these four triplets with no change in the amino acid sequence of the protein specified by the gene in which the triplet occurs. Thus, the nucleic acid sequence can change with no concomitant change in the protein. Theoretically, the nucleic acid sequence can change by 18% while no change is seen in the amino acid sequence of the protein coded for. Nucleic acid hybridization techniques will easily detect a 1% difference between two nucleic acid sequences. The rationale for doing this will be explained below.

In order to compare the RNA sequences of different viruses, nucleic acid hybridization techniques must be used. The basic rationale for the hybridization (or reassociation) reaction is seen in Figure 1 and involves the ability of complementary nucleic acid sequences to recognize each other and interact together. This recognition and interaction leads to the formation of a stable double stranded nucleic acid molecule from two complementary single stranded nucleic acid molecules.

Two virus RNA's can thus be compared by mixing the single stranded RNA of one virus with the radioactive complementary strand from another virus and determining whether a double stranded "hybrid" double stranded molecule can be formed. A hybrid double stranded molecule is one in which one strand is from one virus and the other from a different virus. The radioactive complementary strand to be used for these experiments will consist of a radioactive DNA molecule made from the viral RNA by an enzyme, reverse transcriptase, which uses

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an RNA template to make DNA copies of that template. Recent advances in this area make it possible to obtain a full DNA copy of the RNA molecule. The techniques for forming and assaying for double stranded nucleic acids, DNA:RNA or DNA:DNA are well worked out.

One measure of the degree of relatedness between two viruses is the amount of radioactive DNA (which represents one virus) which will form stable double stranded hybrids with the RNA of the other virus. By proper planning, this parameter can reveal the relative size of the two viral nucleic acids being compared as well as some details of the evolutionary events which have formed the present day virus. It can tell, for example, whether new genetic material has been added or deleted from two related but not identical viruses.

A second measure of the degree of relatedness between two viral nucleic acids depends on the fact that single stranded molecules which are only partially complementary can also interact to form a stable double stranded hybrid molecule as is described in Figures 2 and 3. In this case some of the nucleotides in one strand are paired with a non-complementary (see Figure 3 II) nucleotide in the other strand. The degree of similarity of the two complementary strands of a hybrid molecule can be determined by measuring the thermal stability of the hybrid and comparing it to the thermal stability of a perfectly complementary double stranded molecule (see Figures 2 and 3). Thus, a second measure of the degree of relatedness between two viral RNA's is the thermal stability of the hybrid molecule. As is seen in Figures 2 and 3, the quantitative difference between the complementary sequences of the hybrid molecule can then be determined.

Two parameters, then, define the degree of relatedness between two nucleic acids -- (1) the fraction of the nucleic acid from one virus which will form a hybrid with the RNA of another virus and (2) the thermal stability of the hybrid molecule formed. Different combinations of these two parameters can reveal important events which have occurred during the formation of the present day viruses.

The relationship of the NZB virus to other mouse C-type viruses will also be examined as part of this study.

B. What is the evolutionary history of C-type virus nucleic acid with regard to virus-like DNA present in mouse cell DNA?

The practical reason for doing this study is that it might well provide a framework for determining whether the NZB C-type virus is in fact a "mouse" virus. This type of study has evident importance in these days of hunting for a "human" cancer virus among the C-types.

C-type viruses have been isolated from spontaneously transformed cells of many different species of mammals. In all cases examined thus far, the RNA sequence isolated from a particular virus originating from a particular species are also represented in the DNA

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of the species from which it was isolated. DNA sequences homologous to the viral RNA are also found in non-transformed cell DNA of the same species. It is clear that in at least some of these cases the whole viral genome is present in the normal non-transformed cell since it has been possible to activate "normal" cells and cause them to produce C-type viruses characteristic of that species. These data suggest that the virus nucleic acids are transferred in an inactive form from generation to generation, probably in the germ line. This raises the possibility that these viruses have been present in the mouse during a large part of the "mouse" evolution. We wish to study the evolution of the viral like sequences present in the mouse cells, concentrating on the NZB virus nucleic acid. However, other types of C-type mouse viruses will also be examined.

The methods for doing nucleic acid sequence evolutionary studies are well worked out. The basic technique will involve reacting a very small amount of radioactive viral nucleic acid (the same radioactive DNA used in section A) with a large amount of non-radioactive DNA from a variety of species in order to form hybrid double stranded molecules. In this case the hybrid will be composed of one nucleic acid strand from the virus and one strand from the DNA of a mammal. The non-radioactive DNA will be isolated from a variety of mammalian species which have varying degrees of evolutionary relationship to the mouse. Non laboratory strains of these species will be used where possible. The two parameters of relatedness, (1) the fraction of radioactive viral DNA which is capable of forming hybrids with the non-radioactive mammalian DNA, and (2) the thermal stability of the hybrid molecule, will be measured.

The results of these studies should throw light on the origin of viruses. Further, they may provide us with a framework for judging whether a particular present day virus has been associated with a particular animal species during the evolution of the animal species.

C. How many integrated DNA copies of the NZB C-type virus genome are there per cell in the different NZB cell lines and in different mouse strains?

The results of this study are essential for the proper interpretation of any results obtained from the studies outlined in A and B. The basic technique for determining the copiness per cell of a particular virus nucleic acid is well worked out and has been used to determine the copiness of a variety of viruses in normal and transformed cells. The basis for this type of study involves measuring the kinetics of the formation of double stranded DNA molecules (or reassociation kinetics). The DNA of all mammals is composed of two fractions. One fraction is composed of DNA sequences which are present about 10^5 times per cell and is called the repeated DNA. The second fraction is composed of non-repeated DNA sequences which are present one time per haploid cell. This non-repeated DNA can be used as a calibration standard to determine the copiness of a particular sequence. The time necessary for a DNA sequence to reassociate is directly proportional to the concentration of the DNA sequences present in the reaction mixture. Mammalian DNA at a concentration of 10 mg/ml will reassociate in 1/10 the time that is needed by the same DNA at a concentration of 1 mg/ml. Thus, if a virus sequence is present in a cell at a concentration of 10 copies per haploid cell, it will take 1/10 less time for it to reassociate than it will for non-repeated DNA which is present at a concentration of 1 copy per haploid cell. If, however, a virus sequence is present at the same concentration as the non-repeated DNA sequences, one copy per haploid cell, it will take

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the same time to reassociate as does the non-repeated DNA. Thus, if one measures the time of reassociation of both the non-repeated DNA and the viral sequences, the copiness of the viral sequences can be determined. Viral sequence reassociation can be monitored by utilization of the same radioactive probe described in A and B. The non-repeated DNA reassociation can be followed by using non-repeated DNA labeled with a different isotope. Non-repeated DNA is readily separated from the repeated DNA.

VII. Search for viruses in continuous lymphocyte lines established from patients with systemic lupus erythematosus

As mentioned above, the autoimmune disease of the NZB and (NZB x NZW)_F₁ mice has been the subject of intensive investigation because it is thought to be a very representative model of human systemic lupus erythematosus. In fact, many of the antibodies, which we have suggested are "immunologic tracks" and thus indicate that the host is defending against an RNA tumor virus, are present in these patients. Because of our successful attempt to demonstrate that a virus is present in every continuous lymphocyte line established from the New Zealand mice, a similar study will be carried out in patients with SLE. 25 patients with evidence of circulating antibody against SS DNA or ds DNA will be studied. The lymphocytes from 8 such patients are already in culture. Once established, the lymphoid lines will be studied for the presence of a virus utilizing methods already established for demonstration of the presence of virus in the murine system.

In addition, cell lines will be examined for the presence of complexes of 70S RNA and reverse transcriptase. This latter approach has been reported to be successful for demonstrating a 70S viral RNA associated with a reverse transcriptase in 95% of patients with acute or chronic myelogenous or lymphoid leukemia. If these studies are successful it may be possible to isolate a human tumor virus which would have obvious theoretical, diagnostic, and therapeutic implications.

VIII. Characterization of nucleic acid intermediates of RNA tumor viruses

The fact that antibodies against nucleic acids can be used to detect the presence of nucleic acid intermediates involved in the maturation of RNA tumor viruses opens the possibility that these antibodies could be used to isolate and quantitate the various nucleic acid intermediates involved in the replication of RNA tumor viruses (Tan and Lerner, J. Mol. Biol., 68:107, 1972).

To accomplish this, we plan to pulse label cells from continuously growing NZ murine lymphocyte lines with ³H-thymidine and/or ¹⁴C-uridine either in the presence or absence of 5 µg/ml of actinomycin D. Cells will be disrupted with .5% NP40 and the nuclei removed. This concentration of NP40 does not inhibit antigen-antibody union. The cytoplasm will be incubated at 37° for 30 minutes with 5 µl of human antibody against SS DNA, ds DNA, DNA-RNA hybrids, nuclear RNA, DNA-histone complex

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and anti-pyrimidine dimer. The human 7S antibody which will now hopefully be complexed to nucleic acids will be precipitated by addition of an excess of rabbit anti human IgG. The immune precipitates will be solubilized by treatment with 1% SDS and mercaptoethanol and then extracted with phenol to remove the protein. The nature of the nucleic acids in the precipitate will be then characterized by isopycnic centrifugation in cesium sulfate. In short, this method allows a "1 step" isolation of the intermediates involved in the replication of C type RNA tumor viruses.

In the procedures outlined above, the isolation of any DNA intermediate necessitates the use of antibody which recognizes a particular nucleotide sequence or conformation of the molecule and thus it is possible that certain molecules which any particular antibody does not recognize could be missed. Thus, we have developed a method to "photomark" DNA by induction of pyrimidine dimers. In this way antibody to pyrimidine dimers can be utilized as a generalized reagent to isolate any DNA containing intermediates. Recently other investigators have also demonstrated by fluorescent microscopy the specificity of anti-pyrimidine dimer antibody for DNA in cells which have been exposed to UV irradiation. In agreement with our results this reagent does not react with the DNA of cells which have not been exposed to UV irradiation.

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1. DNA is composed of 4 chemical subunits or nucleotides. A, G, C, T

Long linear polymers of these nucleotides constitute the DNA molecule which in its natural state is in a double strand form.

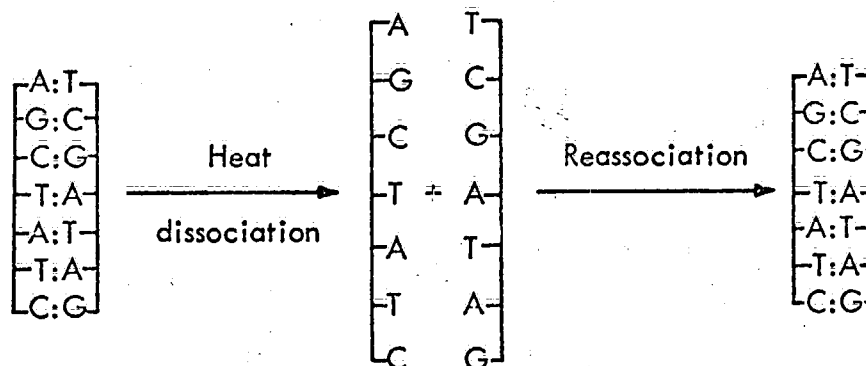
The two strands are held together by specific interaction between the nucleotides in the two strands

A interacts only with T
G interacts only with C

2. In native DNA each nucleotide is paired with its complementary nucleotide in the other strand. The two strands are called complementary strands.

3. Individual strands can be separated by heating. Complementary single strands can reassociate to reform a double strand DNA. A DNA strand can recognize its complementary DNA strand and react with it.

Single Strands



4. The reassociation reaction is a collision dependent reaction. The rate at which double strand molecules reform is then dependent on the concentration of each complementary strand in the solution.

FIGURE 1

THERMAL STABILITY OF DNA

- (1) Strands held together by specific hydrogen bonding between the bases in each strand. The double strand DNA is most thermal stable when all of the nucleotides are properly paired with their complementary nucleotide.



- (2) When all of the nucleotides in the double strand region are not paired with their complementary nucleotide the thermal stability is lowered.

Non-complementary
nucleotide pair



1.5% non-complementary
nucleotide pairs

=

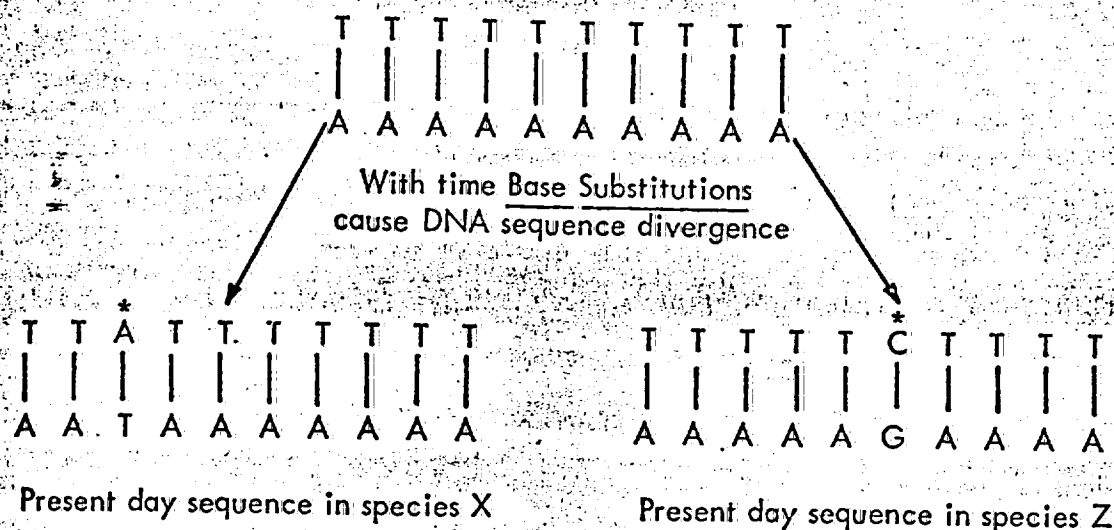
1°C lowering of
thermal stability

- (3) Two single DNA strands can be partially complementary and still reassociate to form a double strand molecule.

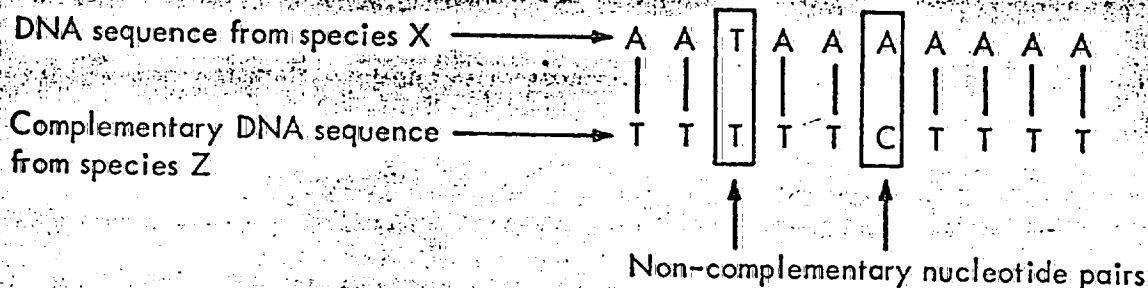
FIGURE 2

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- I. Original DNA sequence in the most recent common ancestor of species X and Z.



- II. The DNA SEQUENCE from species X and Z can be compared in a test tube by forming a HYBRID DOUBLE STRAND DNA molecule. Single-strand DNA sequences do not have to be perfectly complementary in order to reassociate to form a stable double-strand hybrid molecule.



- III. Double-strand DNA is most Thermal Stable when all of the nucleotides in one strand are properly paired with their complementary nucleotide.

When all of the nucleotides in the double-strand region are not paired with their complementary nucleotide, the thermal stability is lowered.

1.5% non-complementary nucleotide pairs = 1°C lowering of thermal stability.

- IV. The percent of non-complementary nucleotide pairs seen for a particular hybrid represents the extent of nucleotide sequence change which has occurred since the time of divergence of the species involved.

*Nucleotide substitution

FIGURE 3

1003538423

11. Publications or papers in press resulting from this or closely related work.

Lerner, R.A., P.J. McConahey and F.J. Dixon. Quantitative aspects of plasma membrane associated immunoglobulin in clones of diploid human lymphocytes. *Science*, 173:60, 1971.

Tubergen, D.G., J.D. Feldman, E.M. Pollock and R.A. Lerner. Production of macrophage migration inhibition factor by continuous cell lines. *J. Exp. Med.* 135:255, 1972.

Lerner, R.A. Relationship of events at the lymphocyte cell surface to gene expression: Approaches to the problem. In: *Cont. Topics in Immunochemistry*, vol. 1, F.P. Inman, ed., Plenum Press, N.Y., 1972, pp. 111-143.

Lerner, R.A., P.J. McConahey, I. Jansen and F.J. Dixon. Synthesis of plasma membrane associated and secretory immunoglobulin in diploid lymphocytes. *J. Exp. Med.* 135:136, 1972.

Kennel, S.J., B.C. Del Villano and R.A. Lerner. Approaches to the quantitation and isolation of plasma membrane associated immunoglobulin. In: *Methods in Molecular Biology*, vol. 6, T. Zacharia, ed., Marcel Dekker, Inc., in press.

Ferrone, S., B.C. Del Villano, M.A. Pellegrino, R.A. Lerner and R.A. Reisfeld. Expression of HL-A antigens on the surface of cultured human lymphoid cells: Effect of inhibitors of protein and nucleic acid synthesis. *Tissue Antigens*, 2:447, 1972.

Lerner, R.A. and F.J. Dixon. The lymphocyte in tissue culture: Its role in biological and genetic studies. *Scientific American*, in press.

Del Villano, B.C. and R.A. Lerner. Expression of a simple "marker" gene during differentiation of the murine teratoma 129 in vitro. In preparation.

Hampar, B., R.A. Lerner, R. Gilden and B.C. Del Villano. Direct syncytial fusion by RD-114 virus in cultured human lymphocytes. In preparation.

Kennel, S.J., B.C. Del Villano and R.A. Lerner. Isolation of plasma membrane associated glycopeptide from continuous cultured murine lymphocytes. In preparation.

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- Del Villano, B.C., S.J. Kennel, A. Puga, R.A. Reisfeld, R.A. Lerner and F.J. Dixon. Characterization of the C type virus of New Zealand Black mice. I. Biochemical properties. In preparation.
- Kennel, S.J., B.C. Del Villano, R.I. Levy, R.A. Lerner and F.J. Dixon. Characterization of the C type virus of New Zealand Black mice. II. Antigenic analysis. In preparation.
- Hall, M.R., W. Meinke, D.A. Goldstein and R.A. Lerner. Synthesis of cytoplasmic membrane associated DNA in lymphocyte nucleus. *Nature New Biology*, 234:227, 1971.
- Tan, E.M. and R.A. Lerner. An immunologic study of the fates of nuclear and nucleolar macromolecules during the cell cycle. *J. Mol. Biol.*, 68:107, 1972.
- Kennel, S.J. and R.A. Lerner. Isolation and characterization of plasma membrane associated immunoglobulin from cultured human diploid lymphocytes. *J. Mol. Biol.*, in press.
- Meinke, W., M.R. Hall, D.A. Goldstein, D.E. Kohne and R.A. Lerner. Physical properties of cytoplasmic membrane-associated DNA. *J. Mol. Biol.*, in press.
- Lerner, R.A., W. Meinke and D.A. Goldstein. Membrane associated DNA in the cytoplasm of diploid human lymphocytes. *Proc. Nat. Acad. Sci.*, 68:1212, 1971.
- Lerner, R.A., M.B.A. Oldstone and N.R. Cooper. Cell cycle dependent immune lysis of Moloney virus transformed lymphocytes: Presence of viral antigen, accessibility to antibody and complement activation. *Proc. Nat. Acad. Sci.*, 68:2584, 1971.
- Hall, M.R., W. Meinke, D. Goldstein and R.A. Lerner. Selective inhibition by rifampicin of the appearance of cytoplasmic membrane associated DNA in diploid human lymphocytes. *Proc. Nat. Acad. Sci.*
- Lerner, R.A., F. Jensen, S. Kennel, F.J. Dixon, G. DesRoches and U. Francke. Karyotypic, virologic, and immunologic analyses of two continuous lymphocyte lines established from New Zealand black mice: Possible relationship of lymphocyte mosaicism to autoimmunity. *Proc. Nat. Acad. Sci.* 69:2965, 1972.

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1003538426

#886 SOBEL

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

December 7, 1972

Grant application No. 886

To: The committee comprising Drs. Andervont, Huebner, and Loosli
Subject: Harold J. Sobel, M.D., Beth Israel Hospital, Passaic, N. J.
New application No. 886
"Effect of Smoke Inhalation of Asbestos Sensitized Hamsters"

3
Denied because
of inadequate
facilities
and
program.

History

This proposal was Case No. 132, and full application was encouraged.

Application No. 886 requests \$31,050 for one year only.

Documents Submitted (attached)

1. Letter from Dr. Sobel dated November 7, 1972
2. Application dated October 4, 1972

Comment

This proposal has been sent to Dr. Arthur Furst for comment; his evaluation is not yet available.

F.W.N.
F.W.N.

FWN:wg
Encls.

1003538427

3736 La Calle Court
Palo Alto, California 94306
(415) 493-9296

January 26, 1973

from the desk of: ARTHUR FURST, Ph.D.

date

to: Dr. Frederic W. Nordsiek
Council for Tobacco Research-USA, Inc
110 East 59th Street
New York, New York 10022

return: yes ☐ no ☐

answer: yes ☐ no ☐

route or cc to:

subject: Comments on Proposal by Dr. Sobel (Case 132)

Applic #886

This proposal is on target, and can yield much needed information on effect of asbestos on animals exposed to smoke.

I should like to caution the investigators about certain pitfalls. This project should be monitored (and if necessary I can consult).

Please note following comments.

1. Chrysotile asbestos used should be UICC standard and must be obtained from Pneumoconiosis Research Unit of Medical Research Council, Llandough Hospital, Penarth, Glam., Wales. Only UICC standards will be acceptable to scientific community.

2. No alteration can be made in the UICC sample sent. Investigator should read Timbrell, V. Powder Technology 5, 279-287 (1971/72). I have reprint in my files. This is in contrast to their reference #6 Badollet of 1965. Also a copy of a personal letter by Dr. Timbrell to me is with Dr. J.H. Kreisher.

3. The investigators should be aware of the dust cloud method for administering asbestos. Timbrell published a simple device (Ann. Occup. Hyg. II, 273-281 (1968). I am not suggesting inhalation over intratracheal administration, but call their attention to the alternate route of administration.

4. I do like the idea of doing analysis on the asbestos for metal content. The UICC standards should come with analytical data.

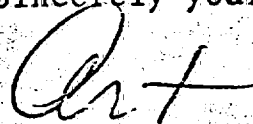
5. Nembutal anesthesia may induce the enzymes which hydroxylate the benzo(a) pyrene. A different brand of Pentobarbital (Diabital) seemed better in our laboratories.

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6. They should be aware of Dantenvill's experiments where he obtained tumors in the respiratory tract other than the lungs using hamsters.

7. When these points are resolved, I feel the project will use a good one.

Sincerely yours,



Arthur Furst, Ph.D.

cc: Drs. Andervant
Hapbner
Hoosli
Gardner
Hockett
Sommers

(also Mar. '73 agenda book)

1003538429

#88L

MAX WACHSTEIN RESEARCH LABORATORIES

BETH ISRAEL HOSPITAL

70 PARKER AVENUE

PASBAIC, NEW JERSEY 07055

HAROLD SOBEL, M. D., DIRECTOR

TEL. (201) 473-8100

November 7, 1972

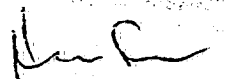
The Council for Tobacco Research, U.S.A., Inc.
110 East 59th Street
New York, N. Y. 10022

Dear Sirs:

Enclosed is my grant application. I am returning it for review by the CTR. I re-reviewed the application, made a few minor corrections and added Dr. William E. Smith's name to the application as consultant.

Dr. Smith is an expert in this area and has agreed to assist us in this work thus strengthening the effort. We will probably be able to obtain the asbestos he used in his studies. This material has been proven a potent cocarcinogen while of itself not being carcinogenic (See reference 3) will assure us a more significant experiment.

Sincerely,



Harold J. Sobel, M. D.
Director of Laboratories

HJS/ejm

1003538430

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

Application For Research Grant

OCT 16 1972

Date: 10/4/72

1. Name of Investigator(s): (include Title and Degrees)

Harold J. Sobel, M. D.* Director of Laboratories
Ruth Schwarz, M. D. Attending Pathologist

2. Institution & Address:

Beth Israel Hospital, 70 Parker Ave., Passaic, N. J. 07055

* Dr. Sobel is also Clinical Associate Professor of Pathology, Columbia University College of Physicians & Surgeons and Special Research Consultant, Zoology & Physiology, Rutgers University

3. Short Title of Project:

Effect of Smoke Inhalation of Asbestos Sensitized Hamsters

4. Proposed Starting Date:

Any time after January 1, 1973

5. Anticipated Duration of this Specific Study:

One year

6. Brief Description of Objectives or Specific Aims:

A. The purpose of this experiment is to determine whether true bronchogenic carcinomas (as evidenced by invasiveness, metastases and ordinary morphologic criteria) resembling those of man can be produced by maximal smoke inhalation in asbestos (chrysotile) sensitized hamsters.

B. In addition we will study the normal and experimentally altered respiratory tract of these animals, using morphologic procedures for the visualization of all cell organelles at the level of the light and electron microscopes; as well as both ultra-structural and histochemical methods for the study of mucopolysaccharides. This work will be undertaken as part of a study presently under way in this laboratory.

C. By subtracting the non-precancerous lesions in the controls from the precancerous ones in the experimental animals (if they develop cancers) and with the special morphologic studies described a better understanding of tumor formation in this system should be forthcoming.

7. Epidermiologic studies indicate that asbestos potentiates the possible carcinogenic effect of tobacco smoke in man (eight times).² The carcinogenic effect of benzo(a)-pyrene (BP) in hamster lung is potentiated by asbestos (chrysotile) which by itself will not produce lung tumors in the hamster.³ An almost 100% incidence of lung tumors can be produced in hamsters with BP and chrys at levels of BP that induce considerably fewer tumors when used alone. Asbestos is an extremely common contaminant of man, and most workers feel that lung cancer is induced by a complex of factors.⁴ From this data it seems logical that the maximal exposure of hamsters to tobacco smoke (using low nicotine high tar cigarettes) following treatment with chrys would be a meaningful way to determine the relative carcinogenicity of tobacco inhalants.

7. Give a Brief Statement of your Working Hypothesis:

See above

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8. Details of Experimental Design and Procedures: (Attach Separate Pages)

We chose the hamster for this work because of their ability to withstand this type of experiment as well as our ability to produce a high incidence of lung tumors in them with chrys and BP.³ We anticipate using a strain that behaved well in a similar study the LVG:LAK male (Lakeview Hamster Colony (now Carworth), Newfield, N. J.).³ We chose not to use mice because of the difficulty in intubating large numbers of these rodents and their ability to filter inhalants well in their upper respiratory passages. The susceptibility of rats to respiratory problems in this type of study and their inability to tolerate asbestos inhalation as well as the slow response of guinea pigs to carcinogens and the anatomic difficulties in intubating them ruled them out for use in this study.

In previous studies asbestos (chrys) controls did not develop lung cancers³ but a virtual 100% incidence of lung cancers could be produced by the additional instillation BP. The incidence at various dose levels of BP was higher when chrys was present. This data suggests that the exposure of hamsters to tobacco inhalants following sensitization by chrysotile instillation would be a meaningful way to determine the relative carcinogenicity of tobacco inhalants. The basis for the use of asbestos as a cocarcinogen are epidemiologic studies indicating that asbestos potentiates the carcinogenic effect of tobacco smoke in man (eight times).² Most workers feel that lung cancer is induced by a complex of factors and it is felt that the common contamination by asbestos fiber inhalation is a significant common

(continued on Page 5)

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

See appended material Page 6.

10. Additional Requirements:

The laboratories are fully equipped and functioning. The only additional requirements are: (1) The CTR smoking apparatus which we understand will be supplied. (2) High tar low nicotine reference cigarettes which we understand will be supplied. (3) Carworth disposable plastic cages which will save personnel time required in maintaining this large number of animals and will in the long run be a saving to the grant. Cost will be \$1,000.

Biographical sketches of all principal and professional personnel (append)

See appended material Page 7 et seq.

12. List of publications: (Five most recent as pertinent) (append)

See references Page 9 et seq.

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13. Budget: (1st year)

A. Salaries (Personnel by names)

Professional

Harold J. Sobel, M. D.
Ruth Schwarz, M. D.
William E. Smith, M.D.

% time

Amount

50%

None

30%

None

Consultant

Technical

Eugene Marquet, M. A.
Animal room technician
Animal room aide

100%

100%

50%

provided by hospital

* Including fringe benefirs

Sub-Total

22,000*

B. Consumable Supplies (list by categories)

Animals
Cage supplies
Food
Chemicals, books, journals

500

1,000

1,500

500

Sub-Total

3,500

C. Other Expenses (itemize)

Illustration and chart preparation
Page costs and reprints
Travel (experimental pathology and cancer meetings
and institutions doing similar work)

250

750

500

Sub-Total

1,500

D. Permanent Equipment (itemize)

None

E. Overhead (15% of A + B + C)

4,050

Total

31,050

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	Not applicable					
Year 3	Not applicable					

It is understood that the applicant and institutional officers
in applying for a grant have read and found acceptable
the Council's "Statement of Policy Containing Conditions
and Terms Under Which Project Grants Are Made."

Signature

Director of Project Harold J. Sobel, M. D.
201-473-8100 EX234 Telephone

Signature

Business Officer of the Institution
David Wachs, Administrator Telephone
201-473-8100 EX221

1003538433

Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current

*

Title of Project

Source

Amount

Duration

None

Pending

Morphochemistry of Aging Alterations in

N.I.H.

\$116,100

** Three years

Drosophila **

* The hospital has subsidized my research by providing me with one full time and one half time pathologist (the department could easily be handled by me or either of my associates with no additional help), research laboratory space equal to that of the routine laboratory or about 10 hospital beds of this 200 bed hospital (see 9), a modern animal room, and secretarial and ancillary personnel. The research laboratory is fully equipped for electronmicroscopic and histochemical work (see 9). In addition the hospital has absorbed occasional small deficits in research funds.

** Direct costs only, competing application

1003538434

denominator.⁴ In fact, in 500 consecutive autopsies of subjects over the age of 15 in both Cape Town, South Africa and Miami, Florida asbestos bodies were found in lung smears of no less than 30 per cent of the males and 20 per cent of the females. The incidence was similar in both cities.⁵

A. Soft chrysotile (approximate length 67 microns) and harsh chrysotile (approximate length 36 microns) will be prepared by the method of Badollet and Gantt⁶ and tested for the presence of nickel, chromium and other potential carcinogens by emission spectroscopy.

The asbestos will be instilled intratracheally with glass pipettes under direct visualization while the animals are under nembutal anesthesia. This procedure has been standardized in our laboratory and was taught us by our consultant, Dr. Smith. Each instillation will be 0.1ml. The animals receiving asbestos will receive twelve weekly injections as follows: 2.5mg harsh chrys X2, then 1.0mg soft chrys X2, then 1.25mg soft chrys X3, then 0.25mg soft chrys X5. There is little difficulty in administering the harsh chrys. The soft chrys must be given in smaller doses to avoid suffocation by the gel formed. We will use a suspension in saline with Tween. The above regime is entirely possible in our hands, and was found to be a potent cocarcinogen in previous work.³

Using the CTR smoking machine and low nicotine high tar cigarettes, we will attempt to maximally smoke the animals so treated. We contemplate adjusting the animals to the smoking machine for a short time prior to the asbestos administration and to continue to smoke them during the interval during which the asbestos is being administered. It may be necessary to somewhat alter the smoking schedule immediately following administration of asbestos.

We plan to use a total of 50 cage controls, 100 sham smoked animals and 100 tobacco smoked animals. Half of each group will be treated with asbestos (Fig. 1). We contemplate including a few extra animals in each of the asbestos treated groups to compensate for the early loss of a small percentage of these animals.

B. As part of an ongoing study in this laboratory tissue from control and experimental animals will be studied. With ordinary histological methods, and with histochemical methods as previously outlined by one of us (H.S.).¹ This system enables us with the light microscope, using relatively large pieces of tissue to distinguish cellular organelles and study their size, number, shape and distribution. The techniques used also provide some biochemical information although quantitative data cannot be obtained with histochemical methods. The information obtained with the light microscope with its wide scope will be searched for with the electron microscope. Some alterations are not found in great frequency, but when noted with the light microscope will be found with the EM if they are searched for diligently. On occasion an EM finding which was thought to correlate with the light cytochemical observation was shown not to do so with EM cytochemical preparations which will also be obtained. This is illustrated in my work with 131_I injury of thyroid ref. 23, 35 and 36.

The characterization of muconolysaccharides by the methods of Spicer including EM methods where applicable should also provide a great deal of insight into the alterations associated with carcinogenesis.

These methods will be used in the differentiation of precancerous from non-precancerous lesions.

C. In future studies asbestos sensitized animals can be used in an attempt to assess the carcinogenicity of other factors.

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9. Facilities Available

The work will be carried out in a well equipped department of pathology. The routine laboratory consists of separate units for hematology, bacteriology, chemistry, blood bank, washroom for glassware, office for secretary, office for associate pathologist and storage space for slides, etc. Space occupied by these units totals approximately 100' X 80'. Rooms which are partially used for research and partially for routine work are the office of the principal investigator (18' X 12') and the room for the preparation of routine histological sections (18' X 12'). Space devoted exclusively to research consists of the electron microscopy laboratory, histochemical laboratory and a 20' X 12' air-conditioned and heat controlled animal room which is able to house at least 400 hamsters and other small animals if required. The electron microscopy laboratory consists of the room used for cutting and preparing tissue sections (18' X 20'), the dark room (8' X 8') and the room housing the electron microscope (9' X 11'). Histochemistry occupies a space 18' X 17'. The electron microscope is an RCA model EMU-3F, there are 2 LKB and 11 Porter-BLUM ultratomes and diamond knives for preparation of ultra thin sections, a high vacuum evaporator, the necessary incubators, pH meters and vacuum pumps in addition to the usual routine equipment such as an autotechnicon, microtomes and knife sharpening machine, automatic glassware washer and a Zeiss microscope with complete automatic photographic outfit. The histochemical laboratory houses a cryostat recently purchased by the hospital, Sartorius freezing microtome, and ample refrigeration space. The dark room contains all necessary tools for the processing of electron micrographs.

Two experienced and very capable associate pathologists have been provided by the institution so that the grant effort will not be unnecessarily interrupted by hospital routine. Secretarial and some animal maintenance personnel are also provided by the hospital

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7.

11. Dr. Sobel's curriculum vitae is attached. He was born in N.Y.C. on R and is R. He was trained as an experimental pathologist at the Mount Sinai Hospital, N.Y.C. (see curriculum vitae) and his major interests are in histochemistry, electron microscopy and autoimmune diseases. He is a frequent reviewer for the Journal of Histo- and Cytochemistry. Dr. Sobel's bibliography is attached.

Dr. Schwarz is a capable, board certified, pathologist who handles the routine pathology at this institution and has sufficient time available to spend 10+ hours in research as well. She was born R. She was trained under Dr. Max Wachstein which is sufficient to explain her abilities in research, and is a graduate of University Lausanne, Medical School. She is co-author with me of my references 43, 45, 47, 48, 57-59, 61-63 and 65 as well as: (1) Wachstein, M. and Schwarz, R.: Occurrence of Hemorrhagic Centrolobular Necrosis in Protein Deficient Rats. Proc. Soc. Exper. Biol. Med. 103, 478, 1960. (2) Wachstein, M., Schwarz, R., and Besen, M.: Electron Microscopy and Enzyme Histochemistry of Tubular Regeneration in Rat Kidney (abstract) Federation Proceedings 23, 546, 1964.

Mr. Marquet is a fine research technician and electronmicroscopist who was born R. He received a B.S. from Queens College (1962) and a M.S. from St. John's University (1965) and has had considerable experience in electron-microscopy in my laboratory and in that of Dr. R. Terry at the Albert Einstein College of Medicine since 1965. He is co-author with me of my references 45-50, 54, 58, 59, 61-63, 65, 67 and 69. He is extremely capable in the design, maintenance and repair of mechanical equipment. He is exceptional in the care of the electron microscope and does remarkable work with radios and automobiles. He built his own home. A very exceptional home. He would be a boon to the CTR and invaluable to the experiment in the use and care of the smoking machine.

Dr. William E. Smith is Director of the Health Research Institute at Fairleigh Dickinson University, Madison, New Jersey. He has extensive experience in experimental carcinogenesis, especially in cancer caused by chemicals and dusts in hamsters, mice and rats and has published some 35 papers in this field. He received his A.B. 1934, Princeton, N.J. and M.D. 1938, John's Hopkins School of Medicine. Dr. Smith was a Fellow in Bacteriology in Harvard Medical School 1938-39, Fellow in Medicine Massachusetts General Hospital 1940-41, Assistant in Bacteriology, Harvard Medical School 1941-43, Assistant in Pathology, Rockefeller Institute 1943-47, Associate Sloan Kettering Institute 1947-49, Assistant Professor 1952-56. His pioneering work and experience with asbestos and lung cancer are a boon to this study.

1003538437

- 8 -
CURRICULUM VITAE

Harold John Sobel, B.A., M.D.

Brooklyn College, Brooklyn, N. Y.

Feb. 1947 to June 11, 1950 - B.A. Cum Laude

The Chicago Medical School, Chicago, Illinois

Sept. 1950 to June 26, 1954 - M.D.

REDACTED

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References for This Work

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FIGURE 1.

Number of Hamsters in each Experimental Group

	<u>No Asbestos</u>	<u>Asbestos*</u>
Cage Control	25	25
Sham Smoked	50	50
Tobacco Smoked*	50	50

* for dosage regimen see 8 (details of experimental design) pages 2 & 5

1003538446

#896 WARREN

1003538447

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

January 18, 1973

Grant Application No. 896

To: The committee comprising Drs. Andervont, Gardner, and Huebner

Subject: Joel Warren, Ph.D., Director, Leo Goodwin Institute for Cancer Research, Fort Lauderdale, Florida
New application No. 896
"Potentiation of Herpesvirus by Tobacco Tar Condensates"

History

This project was Case No. 86, and full application was encouraged.

Application No. 896 requests \$22,994 for one year, with "option of extension" for unspecified amounts.

Documents Submitted (attached)

1. Letter to Dr. Hockett dated January 15, 1973
2. Application dated January 12, 1973.
3. Final report to U. S. Department of Agriculture, December 31, 1972. (Although not marked "confidential", we should doubtless so regard it as it is a report on a contract).

(Also submitted were several reprints and a manuscript which appear not to be relevant, and are not cited under "References" in the application.)

Comment

The statement on "Facilities", item 9 of the application, explains why the name of the institution has changed since receipt of the inquiry which became Case No. 86.

This program appears to be an outgrowth of that of the late Dr. James Reyniers.

F.W.N.

F.W.N.

FWN:wg
Encls.

1003538448

LEO GOODWIN INSTITUTE FOR CANCER RESEARCH

3301 College Avenue, Fort Lauderdale, Florida 33314 Tel. 305 /587-6660

January 15, 1973

**The Council for Tobacco Research
110 East 59th Street
New York, New York 10022
Attn: Robert C. Hockett, Ph. D.
Associate Scientific Director**

Dear Sir:

In November, 1971, we submitted a preliminary inquiry to the Council as to the possibility of support for a study of interaction of tobacco tar and Type 1 herpesvirus. Your Board was kind enough to request that we submit a formal application. However, we delayed such action until we could obtain additional experience with tobacco smoke condensates. For the past 18 months, we have been investigating the ability of tobacco smoke condensate to promote the oncogenic action of avian and murine sarcoma viruses. This work was conducted under a contract with the U. S. Department of Agriculture with Dr. T. C. Tso as Project Officer. A copy of the final report of this study is appended. Unfortunately, it will not be possible to fully interpret and possibly publish this data until the results of five other laboratories investigating these same preparations become available to the USDA. This contract terminates in February, and Dr. Tso has informed us that the outlook for continuing support is poor due to an overall reduction in USDA research funding. A grant from the Damon Runyon Foundation to enable us to investigate the antibody response to herpesvirus in patients with pulmonary carcinoma has also terminated, and we currently have essentially no support to continue research on the possible interaction of tobacco tar and viruses of potential oncogenicity.

A grant application to the National Institutes of Health to enable us to look for herpesvirus fluorescent antigen and/or isolate this agent from human biopsy and autopsy material was submitted in May, 1972, as part of a collaborative package submitted by the University of Miami School of Medicine for \$5.6 million to fund an Institute for Cancer Research. Although no final decision has been made, the

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**A Non Profit Federal Tax Exempt Organization
Contributions are Tax Deductible**

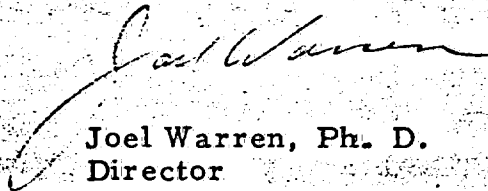
Council for Tobacco Research
January 15, 1973
Page 2

University of Miami has been informed by the National Cancer Institute that the outlook for the proposed institute is bleak and possibly one-fifth of the required funds would be granted.

Should your scientific advisory board require additional information, we will be glad to provide this.

Thanking you for your consideration, I remain

Sincerely yours,



Joel Warren, Ph. D.
Director

Enclosures:
Research Grant Proposal (2)
Publications (5)

1003538450

Dr. Andervont

Dr. Gardner

Dr. Huebner

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET

NEW YORK, N. Y. 10022

Application For Research Grant

JAN 18 1973

Date: Jan. 12, 1973

1. Name of Investigator(s): (include Title and Degrees)

Joel Warren, Ph. D., Director, Leo Goodwin Institute for Cancer Research
Miriam R. Sacksteder, B. S., Research Associate

2. Institution &

Address:

Leo Goodwin Institute for Cancer Research
3301 College Avenue
Fort Lauderdale, Florida 33314

3. Short Title of Project:

Potentiation of Herpesvirus by Tobacco Tar Condensates

4. Proposed Starting Date:

As soon as possible

5. Anticipated Duration of this Specific Study:

One Year with option of extension

6. Brief Description of Objectives or Specific Aims:

This proposal is one of a group of studies into the possible role of HVH-1 in human pulmonary cancer. Concurrent research includes: 1) a study of HVH-1 antibody levels in the sera of normal and cancer patients and their relation (if any) to smoking history; 2) attempts to isolate HVH-1 from normal and neoplastic human lung with the aid of DMSO-BUDR; 3) demonstration of HVH-1 fluorescent antigen in normal and neoplastic pulmonary tissue.

Specific aims of the proposal:

A. We will establish cultures of several cell lines from normal human bronchial and alveolar tissues. These will be maintained as monolayers by established procedures.

B. We will investigate the multiplication of HVH-1 virus in such cultures and whether the presence of tobacco tar condensates in the medium modifies its infectivity.

C. We shall expose human lung cells to tobacco tars in vitro and observe them for evidence of transformation in systems containing live or inactivated HVH-1.

D. The activity of tars known to be carcinogenic in mice and those which promote RSV and MSV will be compared with inactive tars for their effects on human lung tissue and herpesvirus infection.

7. Give a Brief Statement of your Working Hypothesis:

See attached.

1003538451

8. Details of Experimental Design and Procedures: (Attach Separate Pages)

☒ See attached.

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

☒ See attached.

10. Additional Requirements:

☒ None.

1003538452

☒ Biographical sketches of all principal and professional personnel (append)

☒ See attached.

12. List of publications: (Five most recent as pertinent) (append)

☒ See attached.

13. Budget: (1st year)

A. Salaries (Personnel by names)

Professional

	% time	Amount
Joel Warren, Ph. D., Principal Investigator	10%	
Miriam Sacksteder, B. S., Co-Investigator	30%	
Fringe Benefits @ 13%		

REDACTED

Technical

Harriet Jarosz, B. A.	100%	
Fringe Benefits @ 13%		

REDACTED

Sub-Total

\$14,916

B. Consumable Supplies (list by categories)

Tissue culture media, chemicals, sera	2,200
Glassware	350
Photographic and histology reagents	150

Sub-Total

\$ 2,700

C. Other Expenses (itemize)

Hospital travel for specimens	275
Histology services	500
Photographic and graphic supplies	300

Sub-Total

\$ 1,075

D. Permanent Equipment (itemize)

Microscope, binocular, inverted	1,500
---------------------------------	-------

\$ 1,500

E. Overhead (15% of A+B+C)

\$ 2,803

Total

\$22,994

Estimated Future Requirements:

Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
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Year 2 Cannot be established at this time

Year 3

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature

Director of Project and Institute

587-6660

Signature

of the Institution

Administrative Assistant Telephone

Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current

Title of Project	Source	Amount	Duration
Research Involving Employment of Sarcoma Virus to Develop a Short-Term Bioassay System and to Evaluate the Biological Activity of Smoke Condensate	U. S. Department of Agriculture	\$10,800	6/24/71 - 6/23/72
The Role of Herpesvirus hominis, Type 1, in Human Pulmonary Cancer	Damon Runyon Foundation	\$16,000	10/1/72 - 10/31/72
Student Stipend for E. M. Twist	Nova University	2	Annual
General Support, Laboratory Supplies	Leo Goodwin Institute	\$1,000	Annual

ending

Detection and Isolation of Herpesvirus from Normal and Neoplastic Human Pulmonary Tissues	National Institutes of Health	\$20,789	One year.
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7. Working Hypothesis

If one assumes that a viral oncogene is involved in the initiation of human primary lung cancer, then a series of related conclusions follow. The virus (s) should be ubiquitous and persist even in the presence of antibody. Frequent access to the bronchial tree and alveolar membranes from an oropharyngeal habitat would be likely. The agent should belong to a family having oncogenic "relatives" and should be oncogenic and/or teratogenic in experimental systems.

Herpesvirus possesses all of these attributes and has established a commensal relationship with the entire human race and probably all of the higher primates. Approximately 2-3% of asymptomatic adults carry the agent in their oropharynx, and the latent and recurrent characteristics of clinical infection are well known (1). It is estimated that overt illness occurs in only 10-15% of primary infections (2). Clinical recrudescence from the carrier state can be precipitated by a wide variety of physical and chemical stimuli, including trauma (3). Within the past four years, the existence of at least two strains of HVH has been well documented (4). These correlate with the site of virus isolation: Type 1 from non-genital, mainly oral sites, and Type 2 almost exclusively from genital tissues. Transmission of Type 2 appears to be venereal, and its oncogenic role in carcinoma of the cervix is being increasingly substantiated (5,6). The viruses of Burkitt lymphoma, Marek's disease, a primate leukemia and frog kidney cancer, similar to HVH in size and structure, are oncogenic relatives of the herpes group.

Evidence that HVH-1 specifically can be oncogenic is only fragmentary at present. Certain oral strains, "HF" and "JES", induced chromosomal aberrations in tissue culture (7,8). Tanaka and Southam reported that Type 1 strain enhanced development of methylcholanthrene-induced carcinomas in mouse skin (9). We have recently confirmed this (10). HVH was the only virus of several tested found to react with antisera to HeLa cells in vitro (11). Further suggestion of the oncogenic potential of Type 1 strains lies in observations of its teratogenicity in hamsters (12) and the chick embryo (13).

Three of the herpesviruses, MDHV, EBV and HVH are known to exist in a proliferating, stimulatory, non-permissive, and often oncogenic, association with lymphoid cells. HVH can do this in non-natural hosts and characteristically destroys infected cells in vitro soon after infection. Yet the chronic persistence of HVH in man (possibly in sensory ganglia) suggests that it exists in a state of low or non-infectivity under certain conditions.

The potential of non-replicating herpesvirus to be oncogenic has recently been demonstrated by Rapp and his associates (14), who found that hamster embryo fibroblasts underwent malignant transformation when exposed to HVH-2 irradiated by ultraviolet light. The continual involvement of the HSV-2 genome in the neoplasm was demonstrated by immunofluorescence

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and neutralizing antibody in the sera of tumor-bearing hamsters. This resembles the persistence of EBV antigen in cells of Burkitt's lymphoma (15). Whether the persistence of viral genome is required for oncogenicity is not known. Nevertheless, we believe it important to establish whether persistent exposure to low concentrations of tobacco tar, as in the lungs of smokers, could activate latent or infectious herpesvirus (and possibly be an immunosuppressant as well (16) resulting in malignant transformations in the pulmonary tree.

There is considerable literature describing the effect of tobacco smoke or tars on the cell and its nucleic acid in vitro. (For a general review, see Larson and Silvette, ref. 17). However, these studies have been performed primarily with rodent pulmonary tissues. When virus-tobacco smoke combinations were employed, these were investigated in the intact animal (18). The observation that the addition of tobacco "tars" to lung explants induced chromosomal alteration and changes in nuclear DNA (19) encourages us to expect changes in the replication patterns of a DNA virus in the presence of tar. We can find no references to the deliberate combination of HVH and tobacco tar in the same cell system in vitro or in animals although Goldberg, Docherty and Rapp (20) recently demonstrated that 1,2-dimethylbenzanthracene (DMBA) inhibited HVH-2 replication in rabbit kidney cell cultures by blocking synthesis of the viral DNA. Since tobacco tar also contains a variety of polycyclic aromatic hydrocarbons, one could conceive of tar suppressing the cytotoxic effects of HVH-1 in the lungs and yet permitting expression of oncogenicity. Tars will also enhance viral infection and, during the past two years, we have demonstrated that certain tobacco tar condensates are capable of promoting infection by an oncogenic RNA virus, RSV in quail (21). The effect is reproducible with a given tar and related to its composition and mode of tobacco extraction. Freeman, et al., have shown that similar tars will promote the transforming effect of the MSV-0 strain of Moloney sarcoma virus in rat fibroblasts (22). In the light of the preceding discussion, we propose to investigate the question: Can tobacco tar condensates promote or convert HVH-1 into an oncogenic process in lung tissue in vitro?

8a. Procedures

Viruses

Two oral strains of herpesvirus will be used: a recent human isolate, the HSV-1 line received from Dr. B. Roizman, and the older, classical HF strain obtained from Dr. C. Southam. Both strains are currently used in our laboratory for other research. They are maintained in BALB/c mice, Fischer 344 rats, CETC and HEP-2 cell cultures. Standardized virus pools retain stable endpoints at -70° for at least four months when titrated by plaque assay.

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Tobacco Tars

Through the collaboration of Drs. F. Bock (Roswell Park Memorial Institute) and T. C. Tso (U. S. Department of Agriculture), we have obtained 32 experimental cigarette smoke condensates made from experimental flue-cured tobaccos of four entries, each with eight stalk positions. These have been frozen and, when needed, are diluted to a 0.1% solution in DMSO before addition to tissue culture. These same fractions are being tested by Dr. Bock for carcinogenic activity in the mouse skin assay, and his findings could eventually be correlated with our own.

Tissue Culture

Normal, human, fetal and adult, pulmonary and bronchial tissues will be obtained through the collaboration of Dr. R. Poppiti, Department of Pathology, Broward General Medical Center. These will be selected from biopsy and surgical specimens taken from cases of non-malignant disease. The cultivation of human lung tissues has been well described by Sherwin, Richter and Richter (23) and Wellings and Jentoft (24), and the methods proposed are based on their work.

Small (± 0.5 cm or less) tissue fragments will be collected in chilled Eagle's minimal essential medium with double strength antibiotics. Within one hour after transport to the laboratory, they will be repeatedly washed in the above and 1-3 mm explants then placed on a cellophane raft in an organ culture dish in double strength MEM, glucose increased to 130 mg/ml, and 30% fetal bovine serum. When explants are established, they will be minced, trypsinized, and monolayer preparations initiated in Leighton tubes in order to obtain relatively "clean" cultures of epithelial cells. Depending upon results, we plan to select 2 or 3 of the most flourishing lines for the work described below. Because fetal bovine serum has been reported to inhibit spontaneous neoplastic transformation in vitro (25), some culture replicates will be fed with 10% filtered, pooled, human serum obtained from the blood bank of Broward General Medical Center. Since lung tissue monolayers are relatively short lived, (2-6 weeks), we shall periodically go back to explant cultures to renew the monolayer lines.

8b. Experimental Design

Effect of Prolonged Exposure to Tobacco Tars on Transformation of Human Pulmonary Epithelium

Cultures derived from bronchial and alveolar tissues will be serially propagated for several weeks in EMEM containing 0.1 mcg. of different tobacco tar condensates. The latter will be selected on the basis of their plant topography and activity in the mouse skin and sarcoma virus assays. Treated and control cultures will be periodically observed for the following criteria of transformation:

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- 1) Growth of the cells in semi-solid agar.
- 2) Changes in colonial and cell morphology as revealed in Giemsa-stained films and by electron microscopy.
- 3) Changes in the chromosome karyotype.
- 4) Capacity to multiply in the cheek pouch of the hamster (Graffi line) and in the sub-cutis of the irradiated Fischer 344 rat. The rodents will be sacrificed at appropriate times and the fate of the inocula determined by histological examination.

Effect of Tobacco Tars on the Infectivity of HVH-1

Monolayer cultures of human and pulmonary epithelial cells will be infected with graded doses, 1-1000 TCID₅₀%, of both strains of HVH-1 virus in a methylcellulose overlay plaque assay using two plates for each dilution. A similar series of titrations will be performed in cultures containing 0.1 mcg and 1.0 mcg/ml of a tobacco tar condensate. Controls will include non-infected cells and cultures with added tar alone. Four days after infection, a 0.005% solution of neutral red will be added to facilitate plaque counting. This type of experiment will be repeated with each of the 32 available tars. Fluorescent antibody staining for HVH-1 antigen will also be applied to these cultures.

In order to determine whether prior exposure to tobacco tar modifies the response to HVH-1, a series of lung cultures will be exposed to tolerated concentrations of tobacco tars for four days, following which the cells will be transferred to fresh, tar-free media and immediately used for titration of HVH-1. Both cytopathic end points and cell morphology will be compared in treated and control plates.

Will Inactivated HVH-1 Transform Human Lung Tissue and Can This Be Potentiated by Tobacco Tar?

As suggested by the work of Duff and Rapp with hamster embryonic fibroblasts and HVH-2 (14), we shall expose HVH-1 as a 0.5 cm film to a GERT5 ultraviolet lamp for varying intervals to establish its inactivation curve. Following this, we shall prepare irradiated, non-infectious and low infective pools of virus and determine whether the presence of tobacco tar condensates potentiates the ability of HVH-1 to transform human cell cultures. The occurrence or persistence of fluorescent HVH-1 antigen in such cultures would provide an important lead for further exploration. These will also be examined by electron microscopy for the presence of "C" type particles.

Significance of this Research

Prolonged exposure to tobacco tars is a significant pre-disposing factor in the susceptibility to lung cancer. The demonstration of a co-carcinogenic effect of some or all tars on viral inhabitants of the human pulmonary tract could open an important experimental approach to defining the mode of action of tobacco tars as well as explaining the pathogenesis of human pulmonary neoplasia.

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9. Facilities

Nova University, chartered in 1964, is an independent, non-sectarian and non-profit institution for graduate study and research in science and technology and has been accredited by the Southern Association of Schools and Colleges.

The Life Sciences Center, established in 1969, occupies approximately 26,000 sq. ft. in the Louis W. Parker Physical Sciences Building. The physical facilities for the Center were completed in 1970. Laboratories housing the Leo Goodwin Institute for Cancer Research, a component of the Life Sciences Center, occupy a separate wing and have been especially designed to provide air-handling and isolation facilities adapted to the peculiar requirements of gnotobiology. In addition to a large, glass-walled isolator area, there are separate rooms for preparation of the isolators, steam generators, filters and other special equipment. An isolator research area enables the performance of short-term germfree experiments in a location removed from the main laboratory. The animal cubicles permit the holding of mono-infected or normal animals in isolation.

The opposite wing of the Center contains laboratories for research and teaching in molecular biology, virology, immunology and cell culture. It is provided with large incubator and refrigerator rooms, fume hoods, a photomicroscopy laboratory and sterile transfer cubicles. A Philips EM201 electron microscope was installed in 1972.

Supporting services include a well equipped instrument shop, histology and photomicrography laboratories, refrigerated rooms and incubator rooms for cell culture. In the Reyniers Memorial room is a small collection of books particularly concerned with gnotobiology, and the main library which houses a collection of references in the medical and biological sciences is also located in the Farker Building.

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CURRICULUM VITAEJOEL WARRENBirthplace

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Married

REDACTED

Education

- Yale University, A.B. (Zoology), 1936
- Columbia University, A.M. (Bacteriology), 1938
- Columbia University, Ph.D., (Bacteriology), 1940

Positions

REDACTED

Awards

- Order Breasted Cloud and Banner, Chinese National Government, 1946
- Meritorious Service Award, U. S. Department of Defense, 1950

Professional Memberships

REDACTED

Publications

- Approximately 95 in the general fields of microbiology and infectious diseases

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June 1970

CURRICULUM VITAE

MIRIAM R. SACKSTEDER

Date of Birth:

R

Marital
Status:

R

Education:

B.S. Mary Manse College Toledo, Ohio

1949

Positions:

1949-1951

1951-1959

1959-1972

1972-

1971-

Professional
Memberships:

REDACTED

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PULMONARY

599C AVIADO

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 7, 1973

Grant Application No. 599C

To: The committee comprising Drs. Bing, Cattell and Loosli

Subject: Domingo M. Aviado, M.D., University of Pennsylvania School of Medicine, Philadelphia, Pa.
Continuation application No. 599C
"Influence of Cigarette Smoke on Pulmonary Emphysema and Bronchospasm"

History

This applicant has been supported since 1964. The grant now in effect, \$36,775, is for the second and terminal year of a two year project.

The enclosed application, in the amount of \$39,984, plus two additional years, requests "continuation", meaning that it has no priority in competition.

Documents Submitted (attached)

1. Application dated January 29, 1973.
2. Progress report, April 15, 1972 - January 31, 1973.

Comment

Enclosed is a copy of Dr. Bing's report on his site visit, October 18, 1972.

As we informed you a year ago, Dr. Aviado has been on sabbatical. Thus a deceleration of his research is explained. His activities during the year as a CTR Consultant are summarized in the attached copy of a letter to Dr. Hockett dated January 29, 1973.

F.W.N.
F.W.N.

FWN:wg
Encls.

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Comm.

Dr. Bing
Dr. Cattell
Dr. Loosli

CHRONIC PULMONARY DISEASES

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 50TH STREET
NEW YORK, N. Y. 10022
(212) 421-8985

Application for Research Grant
(Use extra pages as needed)

#599C

#599ER1-7/1/72-6/30/73

#599B - 4/1/71-3/31/72

#599A - 4/1/70-3/31/72

#599 - 4/1/67-3/31/70

CF 436 7/1/64-4/1/67

FEB 7 1973

Date January 29, 1973

1. Principal Investigator (give title and degrees).

Domingo M. Aviade, M.D.
Professor of Pharmacology
University of Pennsylvania School of Medicine

2. Institution & address:

University of Pennsylvania School of Medicine
36th Street and Hamilton Walk
Philadelphia, Pa. 19174

3. Department(s) where research will be done or collaboration provided:

Department of Pharmacology
Room 114, Old Medical School Building
University of Pennsylvania School of Medicine

4. Short title of study:

Influence of Cigarette Smoke on Pulmonary Emphysema and Bronchospasm

5. Proposed starting date: July 1, 1973.

6. Estimated time to complete: three (3) years

7. Brief description of specific research aims:

Six objectives are planned. Although most of them will run concurrently for three years, the major emphasis for each six-month period will be one of the following in the order listed:

(a) To determine the mechanism of bronchospasm observed in mice and rats that have been exposed to cigarette smoke for 5 weeks.

(b) To continue the investigation of any role of cigarette smoke in the pathogenesis of pulmonary emphysema in rodents and primates.

(c) To examine the effects of important constituents of cigarette smoke on the bronchomotor, pulmonary vascular, bronchovascular and alveolar structures of the lung. The major constituents that will be studied are nicotine, carbon monoxide, skatole and particulate matter.

(d) To correlate the experimental induction of pulmonary emphysema or bronchospasm with chemical analysis of the lung, with special emphasis on the composition of phospholipid.

(e) To correlate functional changes in the lung with histological and ultrastructural examination.

(f) To develop means of preventing or correcting the abnormalities in function of the lung associated with administration of cigarette smoke or its constituents.

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2.
8. Brief statement of working hypothesis:

Since 1962, the principal investigator has been studying the acute effects of cigarette smoke on dogs, cats, rabbits, hamsters, rats and mice and its chronic effects on the last two species. The results indicate that the bronchomotor system is more sensitive than the pulmonary and bronchial blood vessels to inhalation of cigarette smoke. In the prolonged and repeated administration of cigarette smoke to rats and mice for 5 weeks, there are no signs of pulmonary emphysema and only bronchospasm persists as the most significant functional abnormality. Before discounting the role of cigarette smoking in the pathogenesis of pulmonary emphysema, it is planned to prolong the exposure to cigarette smoke from 5 weeks to 6 months in rodents and to include primates. Since cigarette smoking has been suspected of causing pulmonary emphysema and increase in airway resistance in man, the lesions should be reproducible in animals. It also follows that the mode of action of cigarette smoke on the pulmonary structures can properly be identified in animals. This is our working hypothesis which will be tested during the next 3 years.

9. Details of experimental design and procedures (append extra pages as necessary)

(a) Mechanism of bronchospasm following chronic exposure to cigarette smoke.

In 1968, Ito and Aviado (1) reported a technique for development of experimental emphysema in the rat, which could be used to test agents for provoking or preventing the pulmonary lesions. Although measurements of functional residual capacity did not reveal emphysematous lesions from chronic exposure to cigarette smoke, there was an increase in pulmonary resistance, indicating bronchospasm. During the past year techniques for measuring functional residual capacity, pulmonary resistance and pulmonary compliance were developed in this laboratory (see Progress Report submitted January 31, 1973). Six weeks' exposure to cigarette smoke did not induce pulmonary emphysema but resulted in bronchospasm. Our plan is to determine the mechanism for the development of bronchospasm by testing the influence of parasympathetic blocking agents (such as atropine), ganglionic blocking drugs (such as chlorisondamine) and antihistaminic drugs (such as chlorpheniramine), because previously completed experiments show that in most animal species the short-term inhalation of cigarette smoke stimulated cholinergic and histaminergic receptors in the lung (2). If the concurrent use of blocking agents is unsuccessful, then other mechanisms will be considered, such as release of other bronchospastic humoral agents and structural changes in mucous glands and smooth muscles of the airways. The morphology of the airways will be examined in detail under (e) below.

(b) Pulmonary emphysema in rodents and primates. The long-term exposure of mice and rats will be extended to 6 months. Pulmonary emphysema will be assessed by measurement of functional residual capacity, described under (a) above, and by histological examination, described under (e) below. The details of producing experimental pulmonary emphysema have not been decided. There are no technical difficulties in measurement of functional resistance or pulmonary resistance and compliance, since the techniques used in dogs and cats are applicable. The major problem will be induction of emphysema and we intend to try initially intratracheal ligation and intratracheal injection of papain, which were successfully applied to rodents in inducing emphysema. Our plan is to use 30 monkeys, 150 rats and 300 mice, half of them as controls and the other half to be subjected to daily inhalation of cigarette smoke. The cigarette machine provided by the Council will be used for mice and rats and we plan to develop a special one for monkeys.

(c) Constituents of cigarette smoke. Our previously reported experiments included the testing of acute administration of nicotine (3). We plan to administer nicotine in mist form chronically to mice, rats and monkeys. Since skatole has

9. Details of experimental design and procedures (Continued)

been reported by Carlson *et al.* (9) to produce pulmonary emphysema in cattle and since it is also a constituent of cigarette smoke, this substance will be administered either orally or by inhalation in mice and rats to determine development of bronchospasm and pulmonary emphysema. Carbon monoxide is the third constituent of cigarette smoke that will be tested in mice and rats for chronic action on airways and airspaces. Any long-term effects of nicotine, skatole and carbon monoxide will be investigated for their mode of action in a manner described under section (a) above.

(d) Chemical analysis of lungs and blood. This laboratory has reported techniques for analysis of biogenic amines in the lungs (4) and individual phospholipids (see Progress Report submitted January 31, 1973). The amines relate to the mechanism of bronchospasm and would supplement the observations planned under (a) above. The phospholipids are the basis for surfactant activity of lung extract and may appear concurrently with a reduction in pulmonary compliance, which is likely to occur when the lungs develop pulmonary congestion and edema. Blood analysis for antitrypsin activity, carboxyhemoglobin saturation and gas tensions will be performed by the standard techniques. These measurements will be performed in conjunction with the assessment of lung function described above under (a), (b) and (c).

(e) Histological and ultrastructural examination of pulmonary tissue. The technique described by Loosli *et al.* (10) for removal and fixation of the lungs has been adopted. The estimation of airspaces relative to alveolar walls on a grid will continue. In addition, the principal investigator will collaborate with a pathologist, who will examine the tissue slices by microscope and electron microscope. In conjunction with experiments described in sections (a), (b) and (c), the microscopic examination will allow the localization of early onset of lesions in the airways, blood vessels and alveolar walls. The histochemical technique for identification of fluorescent substances (such as serotonin and catecholamines) developed by Jacobowitz and Koelle (11) will also be applied to the lungs that have been removed from the animals exposed to cigarette smoke and its constituents.

(f) Prevention and correction of abnormalities in lung function. This last aspect of the investigation will be conducted during the final 6 months of the 3-year study. By then, the chronic effects of cigarette smoke will have been identified. In the past, the following drugs have been used either to prevent or to reverse pulmonary malfunction: progesterone against emphysema (5), bronchodilators against bronchospasm (6), naphthoquinones against pulmonary edema (7), and expectorants against deficiency of surfactant (8). Other types of desired actions will be introduced as the pathological processes relating to cigarette smoking are identified.

References Cited Above:

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2. Aviado, D. H. and Palecek, F.: Pulmonary effects of tobacco and related substances; I, II, III: Arch. Environ. Health 15:187-213, 1967.
3. Aviado, D. H. and Samarek, M.: Bronchopulmonary effects of tobacco and related substances; I, II, III, IV: Arch. Environ. Health 11:141-176, 1965.

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References (Continued):

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11. Jacobowitz, D. and Koelle, G. B.: Histochemical correlations of acetylcholinesterase and catecholamines in postganglionic autonomic nerves of the cat, rabbit and guinea pig. J. Pharmacol. Exper. Ther. 148:225-237, 1965.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Laboratories totaling 1,600 square feet are available for this investigation.
They contain the following equipment:

(a) Polygraph and oscilloscopes for measurement of pulmonary resistance and compliance, blood pressures and heart rate; and also artificial respiratory and perfusion pumps.

(b) Spectrophotometer and equipment for paper chromatography.

(c) Gas chromatography apparatus, tonometer for measurement of partial pressure, and Scholander gas analyser.

(d) Fluoroscope for insertion of catheter into cardiopulmonary area.

(f) Microscopes and apparatus for tissue fixation, slicing and staining.

(g) Animal cages.

(h) Special smoking equipment supplied by the Council for Tobacco Research.

11. Additional facilities required:

(Special chambers for administering carbon monoxide, cigarette smoke or aerosol mist from a solution of nicotine or skatole.

12. Biographical sketches of investigator(s) and other professional personnel (append).

10. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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12. Biographical sketches of investigator(s) and other professional personnel:

During the first year, Drs. Belej and Watanabe will continue to collaborate with the principal investigator. During the second and third years, a pathologist and a biochemist will collaborate with him. They will be selected at a future date.

13. Publications:

Aviado, D. M. and Palecek, F.: Pulmonary effects of tobacco and related substances; I, II, III: Arch. Environ. Health 15:187-213, 1967.

Ito, H. and Aviado, D. M.: Pulmonary emphysema and cigarette smoke; experimental induction and use of bronchodilators in rats. Arch. Environ. Health 16: 865-870, 1968.

Aviado, D. M., Sadavongvivad, C. and Carrillo, L. R.: Cigarette smoke and pulmonary emphysema; influence of bronchodilators and biogenic amines in experimental induction in rats. Arch. Environ. Health 20:483-487, 1970.

Aviado, D. M. and Sadavongvivad, C.: Pharmacological significance of biogenic amines in the lungs: 5-hydroxytryptamine, histamine, noradrenaline and dopamine. Brit. J. Pharmacol. 38:353-385, 1970.

Inoh, T. and Aviado, D. M.: Cardiopulmonary effects of progestational agents in emphysematous rats. Chest 59:659-666, 1971.

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CURRICULUM VITAE OF DOMINGO M. AVIADO

Born

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College Education:

University of the Philippines College of Liberal Arts	1940-1942
University of the Philippines College of Medicine	1942-1945
University of Pennsylvania School of Medicine	1946-1948
Doctor of Medicine, University of Pennsylvania	March 1948

Academic Positions at the University of Pennsylvania:

Assistant Instructor in Pharmacology	1948-1949
Instructor in Pharmacology	1949-1950
Associate in Pharmacology	1950-1953
Assistant Professor of Pharmacology	1953-1960
Associate Professor of Pharmacology	1960-1965
Professor of Pharmacology	1965-pres.
Member, Parasitology Graduate Group	1967-pres.

Miscellaneous Positions and Honors:

National Institute of Health Post-Doctorate Research Fellow	1948-1950
Section Editor of Chemical Abstracts	1952-1958
Assistant Attending Physician of Cardiology, Philadelphia General Hospital	1955-pres.
Visiting Lecturer in Anesthesiology, Albert Einstein Medical Center	1955-pres.
Associate Editor of Circulation Research	1958-1962
Visiting Lecturer in Pharmacology, University of the East, R. M. M. C.	1959
Visiting Lecturer in Physiology, Women's Medical College	1961-1962
Travel Award Rockefeller Foundation	1961
Linnaeus Medal, First International Pharmacological Meeting, Stockholm	1961
Fellow of the Guggenheim Foundation	1962-1963
Purkinje Medal, Second International Pharmacological Meeting, Prague	1963
Editorial Consultant, Dorland's Illustrated Medical Dictionary	1963-1967
Consultant, Poison Control Program of Philadelphia	1964-pres.
Visiting Lecturer in Physiology, Rutgers University	1966-1967
Ad Hoc Committee on Air Pollution and Air Hygiene, Philadelphia	
Medical Society, Member	1967-1969
Physician of the Year Award, Philippine Medical Association (Chicago)	1969
Member, Bronchopulmonary Panel, National Clearinghouse for Smoking and Health	1969
Chairman, Medical Advisory Committee, Clinical Research Center, Graduate Hospital of University of Pennsylvania	1969-1970
Member, American Heart Association Ad Hoc Committee on Cigarette Smoking	1969-1970
Editor, "Scapel and Tongs" (Medical Stamps Monthly)	1971-

Societies:

Alpha Omega Alpha Honorary Medical Society: Member	1946
Physiological Society of Philadelphia: Member, 1948, Secretary, 1954-1958; President, 1959-1960; Councillor	1960-1961

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American Society for Pharmacology and Experimental Therapeutics:

Member 1950: Co-Chairman, 1965 Fall Meeting, Member
Finance Committee

1965-1970

American Physiological Society: Member

1951-

American Association for the Advancement of Science

1951

The Society of Sigman XI: Member

1952

John Morgan Society of the University of Pennsylvania, Member
1956, Life member, 1967

American Heart Association: Member 1957: Member Research Study
Committee 1965-1967

**Section on Pharmacology (SEPHAR); International Union of Physiological
Sciences-Treasurer**

1959-1965

International Union of Pharmacology (IUPHAR) Treasurer

1965-1966

American Society of Tropical Medicine and Hygiene: Member

1966

International Leprosy Association: Member

1967

American College of Clinical Pharmacology: Charter Member:

1971

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CURRICULUM VITAE

MIROSLAW A. BELEJ

REDACTED

Date of birth:

REDACTED

Field

Pharmacology

Degree

Ph. D. (June 1971)

Education

1969-1971

THOMAS JEFFERSON UNIVERSITY MEDICAL COLLEGE

Received Ph. D. degree in June 1971. Pharmacology major.
Courses taken: Pharmacology, Toxicology, Drug Metabolism,
Principles of Drug Action.

1965-1968

TEMPLE UNIVERSITY MEDICAL SCHOOL

Received M. S. degree in June 1968. Majored in Pharmacology;
courses included gross anatomy, embryology, histology,
neuroanatomy, physiology, biochemistry, pathology, electro-
physiology, flow dynamics and biostatistics.
Research in cardiovascular area. M. S. thesis published.

1960-1964

TEMPLE UNIVERSITY COLLEGE OF LIBERAL ARTS

Received B. A. degree in February 1964. Biology major.
Among courses taken were mammalian anatomy, comparative
anatomy, animal embryology, histology, physiology,
bacteriology, organic chemistry, qualitative and quantitative
chemistry, and calculus.

Training and
Experience

1971-1972

REDACTED

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Miroslaw A. Belej

1969-1971

1968-1969

REDACTED

Foreign
Languages

Speak, read and write: French and Ukrainian
Read: Russian

Societies

REDACTED

References

Dr. D. M. Aviado, Professor of Pharmacology
University of Pennsylvania
Department of Pharmacology
Philadelphia, Pa. 19104 Tel: (215) 594-8413

Dr. R. W. Manthei, Professor of Pharmacology
Thomas Jefferson University
Jefferson Medical College
Department of Pharmacology
Philadelphia, Pa. 19107 Tel: (215) 829-7969

Dr. J. M. Coon, Professor of Pharmacology
and Chairman of the Department
Thomas Jefferson University
Jefferson Medical College
Philadelphia, Pa. 19107 Tel: (215) 829-7766

Publications

Belej, M. A. et al. The Mechanism of "Nicotine Reversal"
in Phenoxybenzamine-treated Dogs. J. Pharmacol.
Exp. Ther. 164: 342-347, 1968.

Aviado, D. M. and Belej, M. Pharmacology of New Antimalarial
Drugs: Two Quinolinemethanols. Pharmacology 3: 257-
272, 1970

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CURRICULUM VITAE

TETSUYA WATANABE

REDACTED

R

Date of birth: REDACTED REDACTED

Degree D. D. S. (1970)Education

1964-1967 Predental courses taken in Yokohama National University and Kanagawa Dental College.

1967-1971 Received D. D. S. degree on March, 1971 from Kanagawa Dental College. Passed National Board in Dental Medicine on May 1971.

Training and
Experienced

1971 (April-Sept.)

REDACTED

1971-1972

REDACTED

SocietiesPublications

Watanabe, T. and Ito, H. Determination of Inorganic phosphate in presence of G-1-P and Phosphorylase activity. To be submitted for publication.

Watanabe, T. and Ito, H. Quantitative Determination of Phosphorylase Activity in Rat After Disc Electrophoresis on Polyacrylamide Gel and Changes of It's Activity due to the Electrophoresis. Biochim. Biophysica Acta. Submitted for publication.

Watanabe, T., Ito, H. and Cho, Y. Effects of Thyroxine and ADR on cardiac muscle (phosphorylase activity and nucleotides) To be submitted for publication.

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14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

Domingo M. Aviado, M.D.

Professor of Pharmacology

Miraslaw A. Belej, Ph.D.

Assistant Professor of Pharmacology

Tetsuya Watanabe, D.D.S.

Instructor in Pharmacology

% time

Amount

30%

60%

100%

Technical

Henry A. Reutter, Technician

30%

Employee benefits (15% of professional and
8% of technical)

Sub-Total for A

\$25,160

B. Consumable supplies (by major categories)

Animals and animal care

Chemicals and glassware

5,000

1,000

Sub-Total for B

\$ 6,000

C. Other expenses (itemize)

Reprints and publication costs

Travel to attend national meetings

500

500

Sub-Total for C

\$ 1,000

Running Total of A + B + C

\$32,160

D. Permanent equipment (itemize)

Chamber for exposure of animals

Transducers

2,000

1,000

Sub-Total for D

\$ 3,000

E

\$ 4,824

E. Indirect costs (15% of A+B+C)

Total request

\$39,984

15. Estimated future requirements.

	Salaries	Consumable Suppl	Other Expenses	Permanent Equip	Indirect Costs	Total
Year 2	25,160	6,000	1,000	3,000	4,824	\$39,984
Year 3	25,160	6,000	1,000	3,000	4,824	\$39,984

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Bronchovascular Effects of Cigarette Smoke	Council for Tobacco Research (CTR Grant 599)	\$36,775	July 1, 1972 - June 30, 1973
Drug Therapy of Acute Respiratory Insuf- ficiency	Department of the Army (DADA 17-71-C-1060)	\$29,407	April 11, 1972 - March 31, 1973
Cardiopulmonary Toxicity of Propellants for Aerosols	Food and Drug Admin- istration (FDA71-310)	\$54,000	September 1, 1972 - August 31, 1973

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Drug Therapy of Acute Respiratory Insuf- ficiency	Department of the Army	\$37,053	April 1, 1973 - March 31, 1974

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Domingo M. Aviado, M.D.Signature Domingo M. Aviado Date 1/29/73Telephone (215) 594-8413
Area Code Number Extension

Responsible officer of institution

Typed Name _____

Title _____

Signature _____ Date _____

Telephone _____
Area Code Number Extension 13

Checks payable to

Mailing address for checks

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UNIVERSITY of PENNSYLVANIA

PHILADELPHIA 19104

School of Medicine

Undergraduate and Graduate Divisions
DEPARTMENT OF PHARMACOLOGY

(215) 594-8416

January 29, 1973

Dr. Robert C. Hockett
Associate Scientific Director
The Council for Tobacco Research
110 East 59th Street
New York, New York 10022

Dear Dr. Hockett:

I am delivering to your office the original copy and five Xerox copies of my report entitled "Cigarette Smoking and Carbon Monoxide". Copies of the articles are being delivered to Mr. Jenkins for your files.

I am not completely happy with the report. After you have examined it, I would like to make the necessary revisions, additions, or deletion. My original draft was actually longer than the version that is being submitted.

My activities as a Consultant since July 1, 1972 have also included visits to laboratories. I hope to submit a report of my visits sometime in June 1973.

It has been a pleasure to think about carbon monoxide, and I am looking forward to receiving my next assignment.

With best regards,

Sincerely yours,

DMA:ea
Enc.

Domingo M. Aviado
Domingo M. Aviado, MD
Professor of Pharmacology

1003538480

Huntington Memorial Hospital

100 CONGRESS STREET • PASADENA, CALIFORNIA 91105
TELEPHONE (213) 796-0381

October 18, 1972

Site visit

Dr. Aviado

Dr. Aviado was not present, but I was shown around by Dr. Watanabe. The group just received a smoking machine (Walton smoking machine). They have not as yet been able to do much work on smoking and small animals.

On larger animals, as well as on smaller ones, they have measured pulmonary resistance and pulmonary compliance, using small animals such as monkeys, rats, and mice.

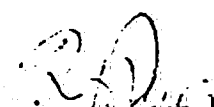
The pulmonary resistance and compliance were measured as usual; the resistance was measured from ratio of pressure over flow, and the compliance from the ratio volume over pressure. In addition, tidal volume, blood pressure, and EKG are recorded.

They are studying the effect of pulmonary edema, which they produce with iodoacetamide and measure the effect of this on these parameters.

Histological studies have not yet been extensively carried out, but they will be done at a later date.

Work on smoking will be done first with low-nicotine cigarettes, and they will measure the functional residual capacity, pulmonary compliance, and airway resistance, together with biochemical studies and blood gases, one month after onset of smoking.

This appears to be a very worthwhile project because of the sophisticated technique and the use of very small and very large animals. I recommend continuation of approval.


Richard J. Bing, M.D.

RJB:bb

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C T R GRANT #599

Progress Report No. 4

April 15, 1972 - January 31, 1973

University of Pennsylvania
Department of Pharmacology
36th Street and Hamilton Walk
Philadelphia, Pa. 19104

BRONCHOVASCULAR EFFECTS OF CIGARETTE SMOKE

Principal Investigator: Domingo M. Aviado, M.D.
Professor of Pharmacology
University of Pennsylvania
School of Medicine
Philadelphia, Pa. 19104

Assisted by: T. Watanabe, D.D.S.
M.A. Belej, Ph. D.

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B. Measurement of pulmonary resistance, compliance tidal volume and total phospholipid content	16
C. Comparison between ICR and Swiss strain mice	18
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ABSTRACT

This Progress Report is being prepared five months prior to the termination of a two-year grant. The conclusions previously reported in a Progress Report submitted April 15, 1972, are as follows:

(a) There are no abnormalities in the bronchial blood vessels in dogs, rats and hamsters repeatedly exposed to cigarette smoke for 4 to 10 weeks. This conclusion was based on measurement of bronchopulmonary blood flow in the dog and on examination of plastic casts of the bronchial vessels of rats and hamsters.

(b) The antitrypsin activity of blood draining the bronchial circulation was found to be equal to that of the systemic blood. The daily inhalation of cigarette smoke by male rats for 10 weeks caused an increase in the level of antitrypsin activity in the blood. There were no functional changes indicating pulmonary emphysema, although rats treated with papain and subjected to tracheoconstriction developed emphysema. The simultaneous administration of cigarette smoke to such rats did not exaggerate the functional changes. In the same series of experiments the rats with pulmonary emphysema experienced a reduction in the level of antitrypsin activity. If a deficiency in this antienzyme relates to the formation of pulmonary emphysema in rats as it does in man, there is no contributing factor due to inhalation of cigarette smoke, since its inhalation caused an elevation in the level of antienzyme activity.

The additional observations completed during the period of April 15, 1972, to January 31, 1973, which are described in this report, are as follows:

(c) Rats that have pulmonary congestion induced by paraquat show an

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elevation of antitrypsin activity of the blood that is not dependent on the adrenal gland. The excised lungs of these animals also show a deficiency in total phospholipid content. Among the 10 phospholipids separated and isolated from the lung extract, the following show a reduction: phosphatidyl choline or lecithin, phosphatidyl ethanolamine, sphingomyelin and lysophosphatidyl choline or lysolecithin. The investigation of the influence of chronic exposure to cigarette smoke on the concentration of each phospholipid is in progress.

(d) It has been possible to measure mechanical properties of the lungs of anesthetized mice. The ICR strain mice have higher values of pulmonary resistance than the Swiss strain. When the ICR strain was exposed twice daily for 5 weeks to smoke generated from low-nicotine cigarettes, there was no change in functional residual capacity, indicating no sign of pulmonary emphysema. However, an increase occurred in pulmonary resistance and a decrease in tidal volume, showing that exposure to cigarette smoke causes chronic bronchospasm.

(e) The Swiss strain mice responded differently from the ICR strain to cigarette smoke. Exposure to low-nicotine cigarette smoke did not increase pulmonary resistance but exposure to high-nicotine cigarette smoke caused an increase. Exposure to either type of cigarette smoke produced a decrease in functional residual capacity. The significance of this reduction is under investigation for the remaining 5-month period during which this grant is in force. Histological examination will be used to determine whether the reduction in functional residual capacity represents pulmonary fibrosis. The development of emphysema indicated by an increase in functional residual capacity can be excluded.

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I. ANTITRYPSIN ACTIVITY OF THE BLOOD AND PHOSPHOLIPID CONTENT OF THE LUNG OF RATS

A continuation of the experiments on the rat consisted of measurement of antitrypsin activity of the blood and analysis of phospholipids in the lung. The results are as follows:

A. Antitrypsin activity

Since inhalation of cigarette smoke produced an increase in antitrypsin activity of the blood, it became important to study another procedure known to produce lung damage. Pulmonary congestion was induced in rats by injection of paraquat (a weed killer), which is known to reduce surfactant activity (Cambar and Aviado, 1970). Paraquat (10 mg/kg) was administered intraperitoneally and 48 hours later the rat was anesthetized and blood collected for analysis of antitrypsin activity according to the technique of Erlanger *et al.* (1961). The results are summarized in Table 1.

The induction of pulmonary congestion by paraquat is accompanied by an increase of antitrypsin activity in the blood. Previous adrenalectomy did not prevent the elevation of antitrypsin activity in response to paraquat. The phenomenon is unrelated to the adrenocortical axis and is not a nonspecific response to stress. The results further suggest that procedures exerted directly on the lung in the form of either congestion (by paraquat) or inhalation (of cigarette smoke) cause an elevation of antitrypsin activity in the blood. One possible intermediate mechanism to explain the end-result is the surfactant activity or phospholipid content in the lung.

(Table 1 appears on the next page.)

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Table 1. Effect of paraquat on antitrypsin activity of blood in rats.

Procedure	No. of Rats	Antitrypsin activity Mean \pm SE (mg trypsin inhibitor/ml serum)
Control	5	1.56 \pm 0.033
Paraquat (10 mg)	7	1.93 \pm 0.092*
Adrenalectomy	3	1.58 \pm 0.018
Adrenalectomy followed by by paraquat (10 mg)	3	1.81 \pm 0.119*

* $p < 0.05$ compared to control rats.

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Table 2. Effect of paraquat on total phospholipid content of lung of rats.

Procedure	No. of Rats	Body Weight Mean \pm SE (g)	Total Phospholipid Mean \pm SE (mg/g wet lung)
Control	4	202.5 \pm 8.5	19.15 \pm 0.73
Paraquat (10 mg/kg)	4	187 \pm 6.6	16.75 \pm 0.99*
Paraquat (20 mg/kg)	4	185 \pm 6.5	15.25 \pm 0.42*

* $p < 0.05$ compared to control rats.

B. Assay of total phospholipids

Three groups of rats were used, as follows: 4 rats received 10 mg/kg paraquat intraperitoneally, and 4 rats 20 mg/kg paraquat by the same route, while 4 rats were controls. After 48 hours the rats were anesthetized with a mixture of urethane (200 mg/kg) and allobarbitol (50 mg/kg) prior to sacrificing the animals for analysis of the lungs. The lungs were minced and homogenized with chloroform-methanol (2:1) mixture and the lipids were extracted by a method described by Folch, Lees and Stanley (1957). The homogenate was filtered through filter paper into a 25-ml graduated glass cylinder and its volume was adjusted to 20 ml. The crude extract was mixed thoroughly with 4 ml of 0.05 % CaCl₂ solution. The mixture was allowed to separate into two phases. After the upper phase was removed, the inside glass wall and the surface of the solution were made clear by the use of the pure solvent upper phase. The resulting lower phase was diluted to 20 ml by the addition of chloroform-methanol (2:1) mixture.

Twenty-five μ l of the extract from the homogenized preparation were digested by heating at 180°C for 30 min in the aluminum heating block, following the addition of 0.6 ml of 70 % perchloric acid. Then water (3 ml), 2.5 % ammonium molybdate (0.5 ml) and 10 % ascorbic acid solution (0.5 %) were added. Color was developed by heating for 5 min in boiling water (at 95°C) according to the method of Rouser, Fleisher and Yamamoto (1970). Optical density was read at 797 m μ by the spectrophotometer. Total lipids were calculated by multiplying the phosphorus value by 25, as reported by Weinstein et al. (1969). The total phospholipid content of 3 groups of rats is summarized in Table 2 (see preceding page).

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The administration of paraquat caused a reduction in the total phospholipid content. The control mean level was 19.5 mg/g wet lung; the rats that received 10 mg/kg paraquat showed a mean level of 16.75 mg/g, and those that received 20 mg/kg paraquat a level of 15.25 mg/g. The reduction in the total phospholipid content correlates with the decrease in surfactant activity of aqueous extract of the lung reported previously (Cambar and Aviado, 1970).

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The phospholipids contained in the lung extract were separated by two-dimensional thin layer chromatography. Eastman silica gel chromatogram sheets without fluorescent indicator were dried before use by heating in an oven for 1 hour at 75° C. Tissue lipids containing 6 or 8 µg phosphorus were applied to the sheet. The sheets were developed in the first dimension with chloroform-methanol-28 % aqueous ammonia-water (65:25:3.2) mixture, and in the second dimension with chloroform-acetone-methanol-acetic acid-water (3:4:1:1:0.5) mixture in a chromatography jar (outside diameter 6 inches, height 12 inches) in a cold room at 4° C. Between runs, the sheets were dried at room temperature in a hood for 30 min and at 75° C in an oven for 1 min. The spots were detected with rhodamine 6G by a technique reported by Marinetti (1962) and Wuthier (1966). The color of the spots and their changes were observed carefully under ultraviolet light. The sheets were cut around the spots and phospholipids were extracted from the sheets with chloroform-methanol (2:1) mixture. The solvent was evaporated in an oven at 75° C in test tubes. Phospholipids were digested with 70 % perchloric acid at 180° C for 30 min and the phosphorus was measured. In the analysis of the small amount of phospholipid, half the volume of the reagent was used.

The results summarized in Table 3 indicate that treatment with paraquat showed a reduction in phosphatidylcholine or lecithin, phosphatidylethanolamine and sphingomyelin. There was also an increase in lyso-phosphatidylcholine (lysolecithin), probably resulting from enzymatic conversion of phosphatidylcholine.

(Table 3 appears on the next page.)

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Table 3. Effect of paraquat on concentration of phospholipids in lung of rats.

Phospholipids	Procedure	Control (4 rats)		Paraquat, 10 mg/kg IP (4 rats)		Paraquat, 20 mg/kg IP (4 rats)	
		% of Total Phospholipid	mg/g Wet Lung	% of Total Phospholipid	mg/g Wet Lung	% of Total Phospholipid	mg/g Wet Lung
Phosphatidylcholine (lecithin)		47.9 ± 1.12	9.21 ± 0.55	46.9 ± 1.35	7.86 ± 0.48	46.8 ± 1.68	7.13 ± 0.25*
Phosphatidylethanolamine		25.9 ± 1.31	4.96 ± 0.28	24.2 ± 0.57	4.05 ± 0.27 ⁺	21.7 ± 1.91	3.32 ± 0.37*
Sphingomyelin		12.5 ± 0.34	2.39 ± 0.08	12.3 ± 0.39	2.06 ± 0.17	12.1 ± 0.46	1.83 ± 0.02*
Lysophosphatidylcholine (Lyso- lecithin)		0.63 ± 0.09	0.12 ± 0.02	1.2 ± 0.13	0.19 ± 0.02 ⁺	1.4 ± 0.22 ¹	0.21 ± 0.03 ⁺
Phosphatidylserine		5.9 ± 0.72	1.12 ± 0.12	6.6 ± 0.77	1.09 ± 0.11	7.2 ± 1.08	1.08 ± 0.13
Phosphatidylinositol		4.1 ± 0.28	0.78 ± 0.03	4.6 ± 1.28	0.76 ± 0.21	6.6 ± 1.18	1.01 ± 0.19
Lysophosphatidylethanolamine		0.50 ± 0.28	0.09 ± 0.05	0.55 ± 0.16	0.09 ± 0.02	0.35 ± 0.15	0.05 ± 0.02
Phosphatidic acid		0.97 ± 0.49	0.20 ± 0.11	0.63 ± 0.26	0.11 ± 0.05	0.65 ± 0.22	0.10 ± 0.03
Diphosphatidylglycerol		0.88 ± 0.21	0.17 ± 0.04	0.83 ± 0.19	0.14 ± 0.03	1.43 ± 0.44	0.22 ± 0.07
Lysobisphosphatidic acid		0.63 ± 0.14	0.12 ± 0.02	0.75 ± 0.10	0.13 ± 0.02	0.95 ± 0.16	0.15 ± 0.03
Unidentified		0.15 ± 0.12	0.03 ± 0.02	1.6 ± 0.64	0.27 ± 0.11	1.13 ± 0.59	0.17 ± 0.09

* Significant difference ($p < 0.05$) compared to control.

⁺ $0.05 < p < 0.1$

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D. Cigarette smoking and phospholipid content

After developing techniques for the separation and analysis of 10 phospholipids the next step was to return to the original problem of cigarette smoking as studied in the rat. These animals are currently being exposed to cigarette smoke daily and the investigation will be completed before June 30, 1973. At that time it is our expectation to correlate changes in blood levels of antitrypsin, the pulmonary content of phospholipids, and measurement of functional residual capacity and pulmonary compliance. We also hope to develop a theory that will show the interrelationship of these factors with the influence of cigarette smoke.

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II. MEASUREMENT OF FUNCTIONAL RESIDUAL CAPACITY AND OF PULMONARY RESISTANCE AND COMPLIANCE IN MICE

It was suggested by Drs Hockett and Kreisher that some of our efforts be devoted to measurement of lung function in mice. If this were possible, then one would be more likely to obtain a breed with a genetic abnormality causing pulmonary emphysema.

The body plethysmograph, endotracheal catheter and intrapleural catheter, which were developed for the rat by Palacek and Aviado (1967), were further reduced in size for use in the mouse. After several trials, we were successful in measuring functional residual capacity, pulmonary resistance and compliance, and respiratory minute volume, which are described in the next paragraphs.

A. Measurement of functional residual capacity

Since functional assessment of pulmonary emphysema depends on measurement of functional residual capacity, the initial step was to develop a procedure that could be repeated in the same mouse. The animal was anesthetized with pentobarbital sodium (30 mg/kg) and a plastic catheter was inserted into the trachea via the oral cavity. The tip was shaped to allow a snag fit inside the lumen of the trachea. Then the mouse was allowed to rebreathe from a syringe containing 5 ml of pure oxygen. After 7 min, the concentration of oxygen in the syringe was determined by a Scholander gas analyzer. The content of nitrogen relates to the volume of air in the functional residual space in the lung, and this capacity was estimated by the following formula:

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$$\text{Functional residual capacity (ml)} = \frac{x(a+b) - 80b}{80 - x}$$

a = volume of syringe (5 ml)

b = volume of tracheal catheter (0.15 ml)

x = concentration of nitrogen in syringe
after 7 min rebreathing (%)

Fifty mice of the ICR strain, ranging in weight from 10 to 40 g and in age from 10 to 60 days, were anesthetized and functional residual capacity was measured. The results are summarized in Figure 1. The functional residual capacity ranged from 0.63 to 1.05 ml. The coefficient of correlation between functional residual capacity and body weight was 0.48.

Each of the 52 mice was allowed to recover from anesthesia for repetition of measurement 2 weeks later. Most of the mice died from bleeding or infection of the trachea. As experience was acquired, the incidence of death was reduced. After 2 weeks 21 mice were still alive for a second measurement. The results are summarized in Table 4. The first group of 7 mice, which had an initial mean weight of 16.7 g, had increased in weight to 25.7 g 2 weeks later. The functional residual capacity was unchanged, with mean values of 0.79 ml and 0.82 ml respectively. The second group of 7 mice were older than the first: the mean weight of 26.7 g had increased 2 weeks later to 32.3 g. There was a reduction in functional residual capacity from 0.85 ml to 0.76 ml after 2 weeks. In the third group, consisting of 5 mice, each showed a reduction in body weight, indicating that these animals had not completely recovered from the first catheterization. There was a fall in functional residual capacity from 0.87 ml to 0.72 ml, a change which was statistically significant.

The death and loss of body weight of some mice following the first

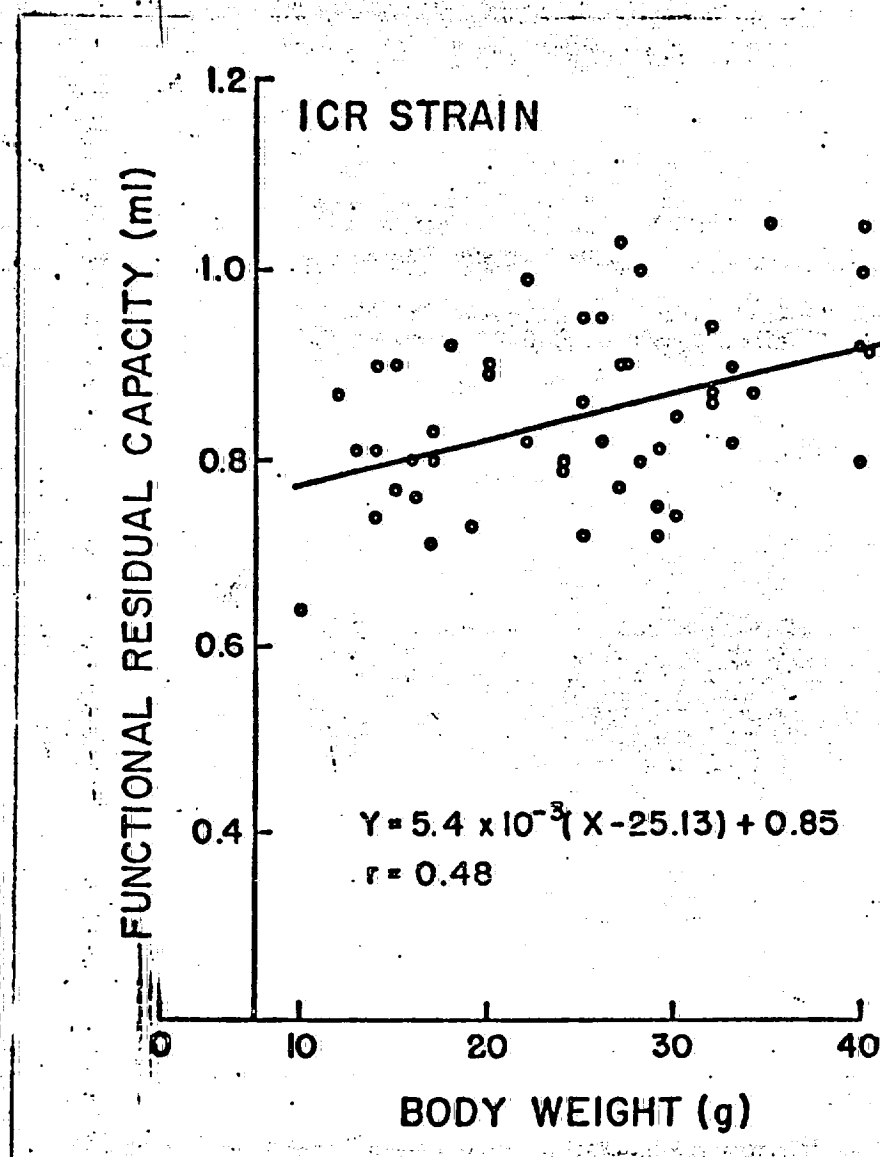
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tracheal intubation discouraged us from continuing the repeated measurement of functional residual capacity. We are developing ^a less traumatic method of insertion of the tracheal catheter. Until this is available, we shall continue our investigation on the basis of a single measurement.

(Figure 1 and Table 4 appear on the next pages)

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Fig. 1. Body weight and functional residual capacity of 50 mice.



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Table 4. Repeated measurements (2-week interval) of functional residual capacity (FRC) of ICR strain of mice.

Mouse No.	First Measurement		Second Measurement	
	Body Wt. (g)	FRC (ml)	Body Wt. (g)	FRC (ml)
1	13	0.81	22	0.82
2	16	0.76	27	0.75
3	17	0.80	24	0.73
4	17	0.83	26	0.83
5	17	0.71	28	0.95
6	18	0.92	27	0.73
7	19	0.73	26	0.94
Mean	16.7	0.79	25.7*	0.82
± SE	± 0.71	± 0.026	± 0.78	± 0.035
8	25	0.95	29	0.74
9	25	0.86	35	0.98
10	26	0.82	32	0.78
11	27	0.66	33	0.65
12	27	0.90	34	0.64
13	28	1.00	30	0.82
14	29	0.75	33	0.74
Mean	26.7	0.85	32.3*	0.76
± SE	± 0.57	± 0.044	± 0.81	± 0.043
15	26	0.95	24	0.66
16	30	0.85	28	0.72
17	32	0.82	33	0.74
18	32	0.87	28	0.76
19	34	0.87	29	0.70
Mean	30.8	0.87	28.4	0.72*
± SE	± 1.4	± 0.021	± 1.4	± 0.017

* $p < 0.05$ compared to first measurement.

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B. Measurement of pulmonary resistance, compliance, tidal volume
and total phospholipid content

Mice of the ICR strain, ranging in age from 10 to 80 days and in weight from 9 to 45 g, were used to measure the mechanical properties of the lung. The body plethysmograph used in the rat was reduced in size for measurement of tracheal air flow, tidal volume, transpulmonary pressure, and pulmonary resistance and compliance. The mouse was anesthetized, the trachea was exposed via a skin incision on the neck, and a plastic catheter was inserted into the trachea and secured with a ligature. After measurements of lung mechanics, the mouse was sacrificed and the total phospholipid content was measured by a technique similar to that applied to the rat (see above). The results are summarized in Table 5. The 30 mice were grouped according to the following weight groups: 9 to 15 g, 16 to 26 g, 27 to 35 g, 36 to 39 g, and 40 to 45 g. As the weight of the animal increased, there was a positive correlation with pulmonary compliance, tidal volume and lung weight, but poor correlation between body weight and pulmonary resistance. The total phospholipid content remained essentially unchanged as body weight increased.

(Table 5 appears on the next page.)

1003538497

Table 5. Relationship of body weight to lung measurements of ICR strain mice.

Mouse No.	Body Weight (g)	Pulmonary Compliance (ml/cm H ₂ O)	Pulmonary Resistance (cm H ₂ O/ml/sec)	Tidal Volume (ml)	Lung Wet Wt. (mg)	Total Phospholipid (mg/g Lung Wet Wt)
51	9	0.026	2.67	0.20	100	--
52	9	0.022	2.94	0.26	97	21.4
53	10	0.029	2.77	0.29	133	18.3
54	11	0.039	2.97	0.33	126	19.2
55	11	0.033	2.67	0.33	100	19.2
56	11	0.055	2.67	0.26	118	19.8
57	12	0.029	2.45	0.30	127	19.0
Mean	10.4	0.0332	2.73	0.28	114.4	19.5
±SE	± 0.43	± 0.004	± 0.068	± 0.017	± 5.7	± 0.43
58	16	0.039	2.67	0.35	176	18.1
59	18	0.046	2.94	0.40	154	18.0
60	21	0.059	2.94	0.45	169	22.7
61	23	0.064	2.67	0.46	174	19.8
62	26	0.078	2.67	0.48	254	20.1
Mean	20.8	0.0572	2.79	0.43	185.4	19.7
±SE	± 1.77	± 0.0068	± 0.066	± 0.024	± 17.6	± 0.85
63	30	0.098	2.45	0.78	236	19.1
64	30	0.098	2.26	0.78	245	21.1
65	30	0.090	--	0.60	210	24.5
66	35	0.117	2.72	0.85	240	22.0
67	35	0.117	2.67	0.78	--	--
Mean	32.0	0.104	2.53	0.76	232.8	21.57
±SE	± 1.22	± 0.0055	± 0.106	± 0.042	± 7.8	± 1.12
68	37	0.133	2.67	1.00	264	22.1
69	37	0.120	2.26	0.98	255	20.4
70	38	0.130	2.67	0.98	282	20.5
71	38	0.145	2.94	0.98	267	19.5
72	39	0.130	2.94	0.91	259	19.3
Mean	37.8	0.131	2.70	0.97	265.4	20.4
±SE	± 0.37	± 0.005	± 0.125	± 0.015	± 4.6	± 0.50
73	40	0.150	2.67	1.01	256	21.7
74	41	0.130	2.67	0.85	310	20.1
75	41	0.145	2.94	--	274	19.2
76	42	0.155	2.94	1.02	239	22.4
77	42	--	2.83	0.87	249	22.0
78	44	0.160	2.26	1.01	350	21.6
79	45	--	--	1.04	284	22.5
80	45	0.150	2.45	0.91	289	21.9
Mean	42.5	0.148	2.68	0.96	281.4	21.4
±SE	± 0.68	± 0.004	± 0.096	± 0.030	± 12.8	± 0.41

1003538498

C. Comparison between ICR and Swiss strain mice

The next step was to measure the mechanical properties of the lung of Swiss strain mice. Twenty-four mice were used, ranging in weight from 10 to 45 g. The results are summarized in Table 6. As the body weight increased, there was a rise in pulmonary compliance, tidal volume and lung wet weight. Pulmonary resistance and the total phospholipid content of the lung did not increase with the other parameters.

The results of experiments on ICR and Swiss strain mice are summarized in Figures 2 to 5. The coefficients of correlation between body weight and each of the other parameters have been calculated. There is a good correlation between body weight and each of the following factors for both strains: tidal volume, pulmonary compliance and wet weight of the lung, but ^{poor} correlation with pulmonary resistance. The following difference exists between the two strains of mice: pulmonary resistance for the ICR strain is higher than that for the Swiss strain.

(Table 6 and Figures 2 to 5 appear on the next pages)

1003538499

Table 6. Relationship of body weight to lung measurements of Swiss strain mice.

Mouse No.	Body Weight (g)	Pulmonary Compliance (ml/cm H ₂ O)	Pulmonary Resistance (cm H ₂ O/ml/sec)	Tidal Volume (ml)	Lung Wet Wt. (mg)	Total Phospholipid (mg/g Lung Wet Wt)
81	10	0.020	1.70	0.20	100	18.8
82	12	0.026	1.23	0.42	123	21.1
83	13	0.036	1.40	0.29	135	22.6
84	14	0.042	1.34	0.36	135	20.4
85	15	0.040	1.47	0.38	120	26.6
Mean	12.8	0.0328	1.42	0.33	122.6	21.9
±SE	± 0.86	± 0.004	± 0.079	± 0.039	± 6.4	± 1.32
86	23	0.078	1.40	0.45	163	20.5
87	25	0.056	1.80	0.40	210	21.7
88	26	0.070	1.40	0.65	185	22.7
89	27	0.095	1.96	0.40	220	25.0
90	28	0.084	2.04	0.65	--	--
91	29	0.084	1.60	0.65	235	24.6
Mean	26.3	0.0778	1.70	0.53	202.6	22.9
±SE	± 0.88	± 0.005	± 0.113	± 0.053	± 12.8	± 0.85
92	30	0.080	1.23	0.46	234	25.0
93	30	0.117	1.96	0.71	--	--
94	31	0.088	2.10	0.67	240	27.9
95	32	0.085	1.47	0.65	328	23.9
96	32	0.117	1.96	0.59	--	--
97	33	0.100	1.55	0.65	250	21.2
98	35	0.098	1.34	0.71	279	20.4
99	35	0.110	1.70	0.78	260	23.3
Mean	32.2	0.0994	1.66	0.65	265.2	23.5
±SE	± 0.70	± 0.005	± 0.113	± 0.034	± 14.1	± 1.04
100	36	0.120	1.84	0.59	280	24.6
101	37	0.130	1.40	0.97	311	21.8
102	38	0.170	2.04	0.97	245	23.2
103	41	0.130	1.96	1.05	310	23.5
104	45	--	--	1.00	350	20.0
Mean	39.4	0.138	1.81	0.92	299.2	22.5
±SE	± 1.63	± 0.011	± 0.143	± 0.083	± 17.5	± 0.72

1003538500

Fig. 2. Pulmonary resistance and body weight of I C R and Swiss strain mice.

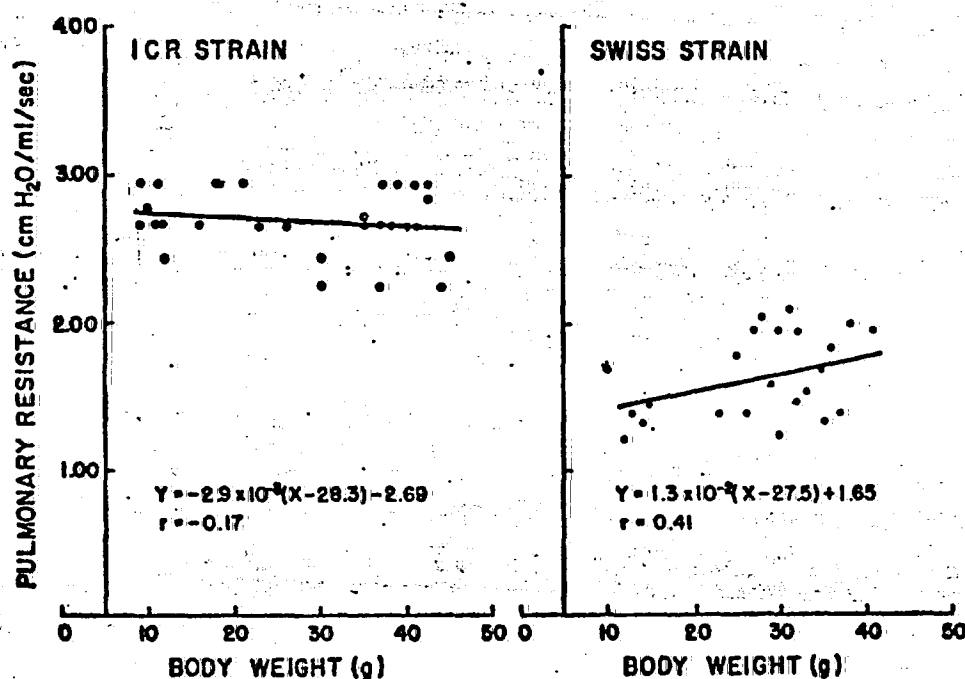
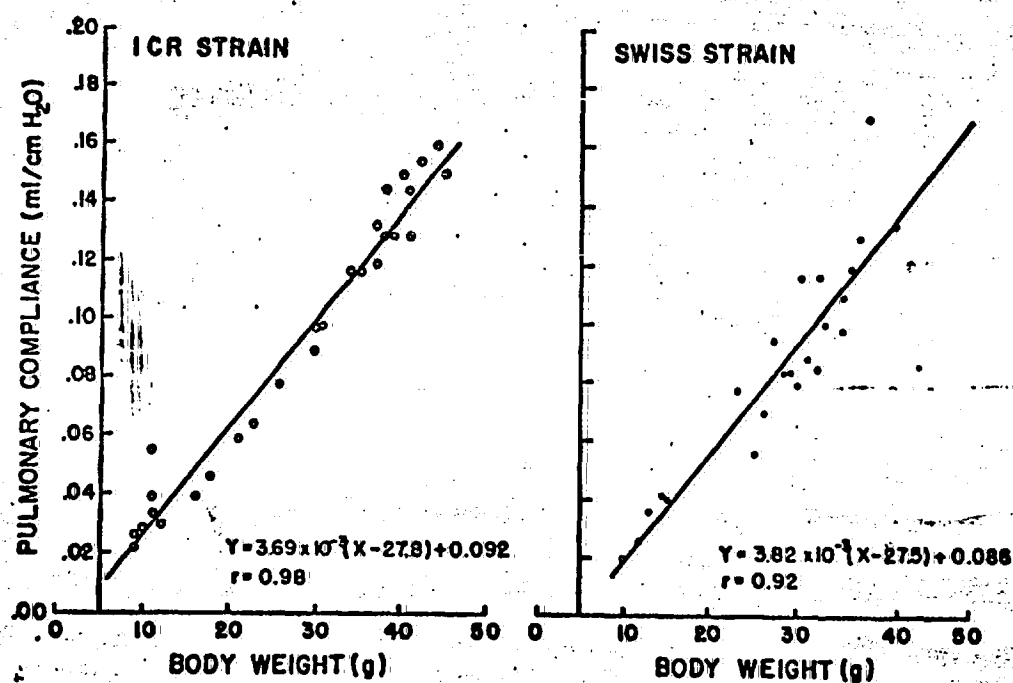


Fig. 3. Pulmonary compliance and body weight of I C R and Swiss strain mice.



1003538501

Fig. 4. Tidal volume and body weight of I C R and Swiss strain mice.

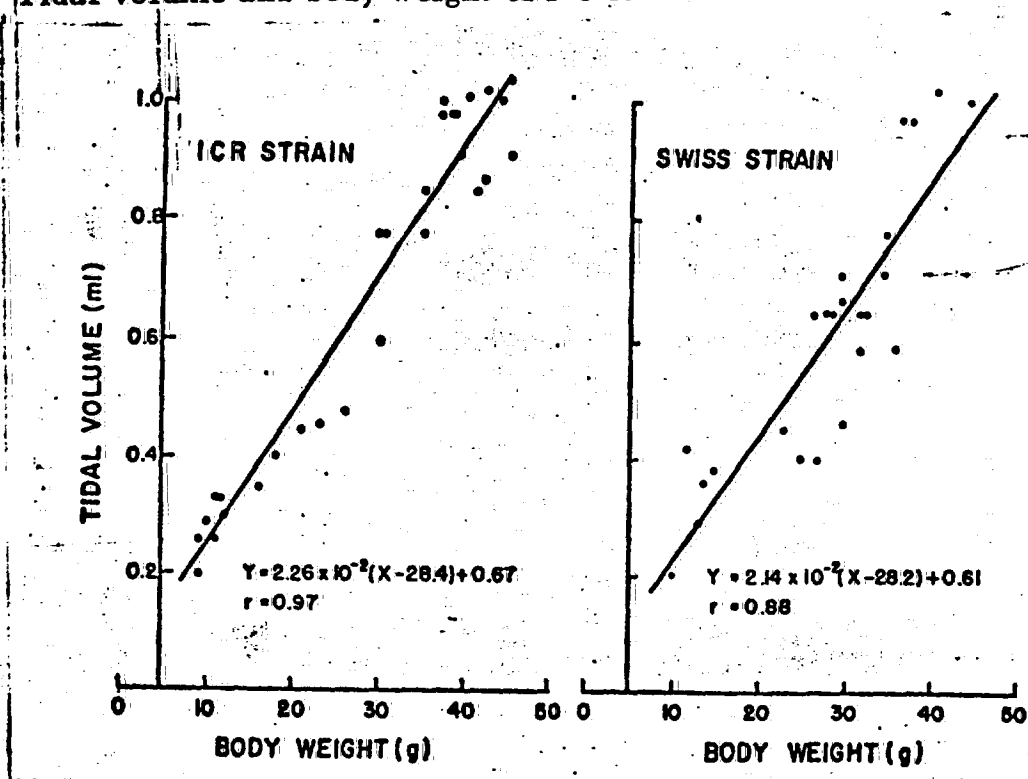
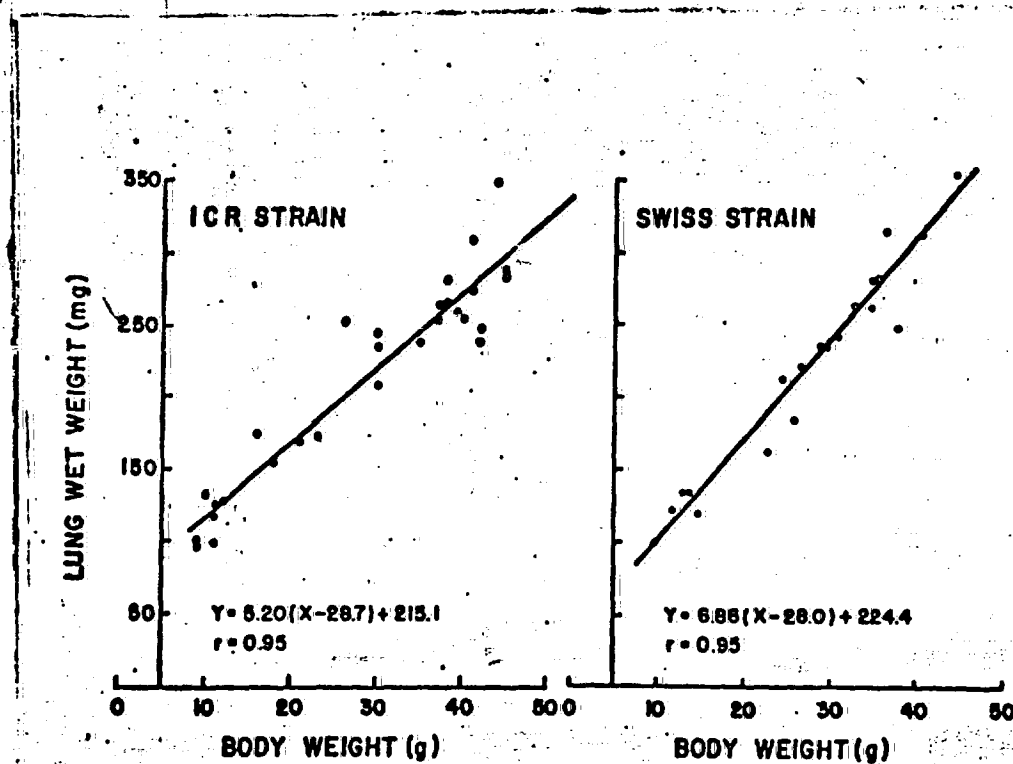


Fig. 5. Lung wet weight and body weight of I C R and Swiss strain mice.



1003538502

D. Daily inhalation of cigarette smoke for five weeks by ICR strain mice

The smoking machine was used to expose mice to cigarette smoke. The mice were conditioned to the machine twice daily for 1 week. Each period of exposure to the machine lasted for 8 min. Then the mice received 8 min (or 8 puffs) of smoke generated from 2 lighted cigarettes twice daily for 5 weeks. The University of Kentucky standard cigarettes were used and were of 2 types according to nicotine content. Analyses of the leaf tobacco for the 2 types were respectively as follows:

Type I A I: 0.31% nicotine

Type I R I: 2.09% "

The initial use of the smoking machine was for a group of 8 ICR strain mice, initially weighing 25 to 30 g. These were exposed to cigarette smoke generated from I A I cigarettes, made with tobacco leaf having a low nicotine content. A control group consisting of 8 ICR mice, was not exposed to cigarette smoke. The results, summarized in Table 7, indicate that exposure to cigarette smoke did not influence functional residual capacity, pulmonary compliance, total phospholipid content, or the ratio of lung weight to body weight. However, there was a reduction in tidal volume and an increase in pulmonary resistance in the mice exposed to cigarette smoke, as compared with the control mice. The extent of the reduction was as follows: tidal volume from 22.0 ± 1.39 ml/kg for controls and 19.1 ± 1.3 for mice exposed to cigarette smoke; pulmonary resistance from 2.61 ± 0.057 cm H₂O/ml/sec for controls and 2.83 ± 0.148 for mice exposed to cigarette smoke.

(Table 7 appears on the next page.)

1003538503

Table 7. Influence of exposure of ICR strain mice to smoke generated from low-nicotine cigarettes twice daily for five weeks.

Procedure	Mouse No.	Body Weight (g)	Functional Residual Capacity (ml)	Pulmonary Compliance		Pulmonary Resistance (cm H ₂ O/ml/sec)	Tidal Volume (ml)	Lung Wet Wt	Total Phospholipid (mg/g Lung Wet Wt)
				(ml/cm H ₂ O)	(ml/cm H ₂ O/kg)			Body Wt (mg/g)	
Control	105	34	0.87	0.087	0.25	2.45	15	7.9	21.1
	106	33	0.88	0.078	0.24	2.83	21	9.8	24.5
	107	24	0.83	0.057	0.24	2.63	19	8.2	23.0
	108	30	0.85	0.075	0.25	2.83	26	7.4	22.0
	109	30	0.93	0.070	0.23	2.67	26	7.1	20.9
	110	30	0.85	0.078	0.26	2.45	20	7.5	--
	111	29	0.95	0.065	0.22	2.45	25	7.0	--
	112	30	0.90	0.072	0.24	2.53	24	6.4	--
	Mean	30.0	0.88	0.073	0.24	2.61	22.0	7.7	22.4
	±SE	± 1.1	±0.015	± 0.003	±0.004	± 0.057	± 1.39	±0.36	± 0.66
Low-nicotine cigarettes	113	28	1.42	0.084	0.30	2.37	23	7.3	--
	114	32	1.10	0.078	0.25	3.34	21	7.9	21.0
	115	25	0.66	0.072	0.29	2.84	13	8.0	22.4
	116	32	0.56	0.070	0.22	3.20	14	6.9	22.0
	117	33	0.92	0.052	0.16	3.20	20	6.5	21.6
	118	21	0.94	0.061	0.21	2.37	22	8.2	22.5
	119	21	0.83	0.061	0.21	2.33	19	--	--
	120	36	--	0.086	0.24	2.96	21	--	--
	Mean	28.5	0.92	0.071	0.23	2.83 ⁺	19.1 ⁺	7.5	21.9
	±SE	± 2.0	±0.11	±0.004	±0.016	±0.148	± 1.30	±0.28	± 0.28

⁺ 0.1 < P < 0.2 compared to control mice.

1003538504

E. Comparison of the effects of low-nicotine and high-nicotine cigarettes in Swiss strain mice

The exposure of Swiss strain mice to cigarette smoke elicited reactions that were different from those in the ICR strain. The results shown in Table 8 indicate that exposure for 5 weeks to IAI (low-nicotine) cigarettes did not influence pulmonary resistance, tidal volume, pulmonary compliance, phospholipid content or the ratio of lung wet weight to body weight. Unlike the ICR mice that showed an increase in resistance and a decrease in tidal volume, the Swiss^{strain} mice did not show any such effect.

The measurement of functional residual capacity indicated a reduction in Swiss^{strain} mice exposed to smoke generated by IAI cigarettes. The control group had a mean value of 1.54 ± 0.17 ml and the exposed group one of 1.03 ± 0.16 ml. The difference between the 2 groups was significant statistically ($p < 0.05$ level).

A third group of Swiss strain mice was exposed twice daily for 5 weeks to smoke generated by IRI cigarettes, which have a higher nicotine content than the IAI type. The rats exposed to high-nicotine cigarettes showed the following differences from control rats: lower functional residual capacity, higher pulmonary resistance and lower compliance if expressed in absolute terms. However, the compliance/body weight ratio was unchanged, and tidal volume, total phospholipid lung content and wet weight/body weight were not different from those of the control group of mice.

(Table 8 appears on the next page.)

1003538505

Table 8. Influence of exposure of Swiss strain mice to smoke generated from low-nicotine or high-nicotine cigarettes twice daily for five weeks.

Procedure	Mouse No.	Body Weight (g)	Functional Residual Capacity (ml)	Pulmonary Compliance (ml/cm H ₂ O)	Pulmonary Compliance (ml/cm H ₂ O/kg)	Pulmonary Resistance (cm H ₂ O/ml/sec)	Tidal Volume (ml)	Lung Wet Wt Body Wt (mg/g)	Total Phospholipid (mg/g Lung Wet Wt)
Control	121	30	1.30	0.066	2.2	1.96	24	7.1	20.7
	122	33	1.58	0.069	2.1	1.84	18	8.4	27.6
	123	30	1.55	0.091	3.0	2.05	24	8.1	24.6
	124	32	1.23	0.061	1.9	1.96	18	8.0	23.2
	125	28	1.65	0.065	2.3	2.19	23	7.8	21.8
	126	32	0.71	0.065	2.0	1.56	20	7.1	23.2
	127	32	1.36	0.098	3.1	1.67	18	7.0	22.7
	128	31	1.80	0.059	1.9	1.13	21	7.1	26.5
	129	33	2.15	0.065	2.0	1.55	20	6.3	25.0
	130	34	1.17	0.091	2.7	1.63	31	7.0	--
	131	29	2.99	0.056	1.9	1.23	18	--	--
	132	30	1.00	0.048	1.6	1.47	14	--	--
	Mean	31.2	1.54	0.0695	2.22	1.69	20.8	7.39	23.9
	±SE	± 0.52	± 0.17	± 0.0028	± 0.14	± 0.09	± 1.26	± 0.21	± 0.74
Low-nicotine cigarettes	133	28	0.85	0.074	2.6	1.96	23	8.1	26.6
	134	31	1.15	0.065	2.1	2.10	19	8.6	20.4
	135	30	0.71	0.081	2.7	1.91	28	6.5	22.5
	136	30	0.83	0.072	2.4	1.55	32	7.3	22.5
	137	32	1.61	0.065	2.0	1.44	20	7.9	18.0
	Mean	30.2	1.03*	0.0714	2.36	1.79	24.4	7.7	22.1
	±SE	± 0.66	± 0.16	± 0.003	± 0.13	± 0.13	± 2.46	± 0.36	± 1.31
High nicotine cigarettes	138	23	1.24	0.039	1.7	1.70	16	9.3	21.2
	139	26	0.73	0.085	3.3	2.45	29	7.4	25.0
	140	25	0.57	0.039	1.6	1.84	15	7.6	27.4
	141	29	0.95	0.078	2.7	2.10	18	6.2	23.9
	142	33	1.03	0.065	2.0	1.96	15	7.8	23.3
	143	26	--	0.052	2.0	2.45	22	6.9	23.7
	144	28	--	0.065	2.3	2.26	19	--	--
	145	27	--	0.065	2.4	2.45	22	--	--
	Mean	27.2*	0.90*	0.0610*	2.25	2.15*	19.5	7.5	24.1
	±SE	± 1.06	± 0.12	± 0.0049	± 0.20	± 0.11	± 1.68	± 0.42	± 0.84

* p < 0.05, compared to control mice.

+ 0.05 < p < 0.1 compared to control mice.

1003538506

F. Histological examination of the lung

One lobe of each animal that has been sacrificed in the above-mentioned experiments has been removed and preserved according to the technique of Loosli et al. (1970). We are awaiting the completion of the following experiment that is still in progress: ICR mice exposed 2 x daily for 5 weeks to high-nicotine cigarettes. When the latter experiment is completed, it would allow a comparison of the reactions of ICR and Swiss strain mice in response to low-nicotine and high-nicotine cigarette smoke, administered twice daily for 5 weeks. After completion, the lung samples will be examined by an expert pathologist who will have no information on the past history of each sample.

1003538507

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1003538508

764B COCHRANE

1003538509

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

February 6, 1973

Grant Application No. 764B

To: The committee comprising Drs. Jacobson, Loosli and Wyatt

Subject: Charles G. Cochrane, M.D., Scripps Clinic and Research
Foundation, LaJolla, California
Continuation application No. 764B
"The Mediation of Inflammatory Injury of Tissue"

History

The grant now current, in the amount of \$31,269, supports the last year of a three year program.

The present application, in the amount of \$37,892, plus two additional years, requests "continuation", but has no priority in competition.

Documents Submitted (attached)

Application dated January 30, 1973, including progress report, biographies and bibliographies.

Comment

The question here appears not to be of excellence, but rather of relevance.

F.W.N.

F.W.N.

FWN:wg
Encls.

1003538510

Comm.

Dr. Jacobson

Dr. Loosli

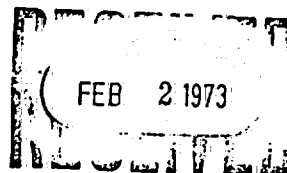
Dr. Wyatt

CHRONIC PULMONARY DISEASES

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A.

110 East 59th Street
New York, N. Y. 10022

Application For Research Grant



Date: January 30, 1973

#764B

#764R2-7/1/72-6/30/73

#764R1-7/1/72-6/30/72

#764S - 9/70

#764 - 7/1/70-6/30/71

1. Name of Investigator(s): (include Title and Degrees)

Charles G. Cochrane, M. D. Member

Peter M. Henson, Ph. D., Associate Member

Stephen W. Russell, D. V. M., Ph. D. - Research Fellow

Thomas C. Kravis, M. D. - Research Fellow

2. Institution & Address:

Department of Experimental Pathology
Scripps Clinic and Research Foundation
476 Prospect Street
La Jolla, California 92037

3. Short Title of Project: The Mediation of Inflammatory Injury of Tissue

4. Proposed Starting Date: July 1, 1973

5. Anticipated Duration of this Specific Study: 3 years

6. Brief Description of Objectives or Specific Aims: The proposed studies will examine the mechanisms by which inflammatory injury of tissue occurs. Both humoral and cellular factors will be examined:

1. To study the mechanism of activation of the kinin forming, intrinsic clotting and fibrinolytic systems. In the previous three years of support by the CTR, the initial components of each of these systems has been isolated in highly purified form in the native (precursor) state from human and rabbit plasma. These components have been radiolabelled and antibodies have been prepared against each. We are now in a position to examine in detail the participation of these systems in inflammatory injury of tissue.

2. Studies of inflammatory injury mediated by leukocytes and other cells. The participation of cellular reactants in inflammatory injury will be studied. The activation, mechanism of release and modulation of these phenomena will be studied.

7. Give a Brief Statement of your Working Hypothesis: Inflammatory injury, induced by a wide variety of stimuli, is effected by a series of humoral and cellular mediators. While in many instances the causative inciting agents remain unknown, the mediation systems of the injury are common to them all and subject to analysis. Through analysis and understanding of these systems, inhibitors of the sequential injurious events can be sought and disease prevented.

1003538511

8. Details of Experimental Design and Procedures: (Attach Separate Pages)

Scientists have undertaken two general approaches to the problem of inflammatory injury of tissue, the first involving a search for inciting agents that initiate the disease such as microorganisms, chemical agents, specific antibodies, etc., and the second, involving the analysis of the host factors, brought into the arena by the inciting agent, that are responsible for mediating the structural injury of tissue. These host factors include the humoral and cellular mediation systems. In many inflammatory diseases currently under investigation the inciting agents remain an enigma. Such is the case in rheumatoid arthritis, certain forms of pulmonary fibrosis, thyroiditis, multiple sclerosis, polyarteritis nodosa, to name only a few diseases. Single or combined inciting agents have not been detected consistently despite a valiant effort. The reasons for this are complex and possibly specific to each disease. (continued on page 5)

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

Approximately 2000 sq. ft. of laboratory space is available. The laboratory is equipped with refrigerated centrifuges, electrophoretic and chromatographic equipment, five fraction collectors, a walk-in cold room, deep freezes (-20° and -70°), complete fluorescent equipment and a preparative ultracentrifuge. Histologic and electron microscopic analyses are carried out in facilities available and frequently used by the applicants. An analytical ultracentrifuge and amino acid analyzer are also used by the applicants within the department. Complete isotope labeling, hot lab and monitoring facilities are in current use. Two Schultz-Dale bath apparatuses are used for assays of biologically active materials.

10. Additional Requirements:

None

11. Biographical sketches of all principal and professional personnel (append)

appended

12. List of publications: (Five most recent as pertinent) (append)

appended

1003538512

13. Budget: (1st year)

A. Salaries (Personnel by names)

Professional

	% time	Amount
Charles G. Cochrane	20	-0-
Peter M. Henson	20	-0-
Stephen W. Russell	50	8,000

Technical

Lynette Buettner	100	7,500
Dishwasher	30	2,000
Animal Caretaker	30	2,200
Sub-Total		19,700

B. Consumable Supplies (list by categories)

Chemicals, proteins	5,200
Glassware, plasticware	5,200
Animals, feed and bedding	1,500
Sub-Total	11,900

C. Other Expenses (itemize)

Travel, 1 fellow to East Coast meeting	450
Reprints	900
Sub-Total	1,350

D. Permanent Equipment (itemize)

E. Overhead (15% of A+B+C)

4,942	
Total	37,892

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	20,100	11,900	1,350		5,002	38,352
Year 3	20,600	11,900	1,350		45,077	38,927

Signature

Charles G. Cochrane

Director of Project (714) 459-2390

Signature

Edward K. Kenney

Business Officer of (714) 459-2390

the Institution

OK

1003538513

Other Sources of Financial Support

List financial support for research from all sources,
including own institution, for this and/or related research projects

Current

Title of Project	Source	Amount	Duration
Immunologic Studies	United States Public Health Service	150,000	1973-74
The Mediation of Inflammatory Injury of Tissue	Council for Tobacco Research	31,269	1972-73

(This grant supports entire costs of research of seven full time investigators - including the three applicants, eight technicians, two animal caretakers, a glassware washer and one secretary)

Pending

None

1003538514

8. Details of Experimental Design and Procedures: (Continued) - 2

While efforts must continue to gain an understanding of the inciting agents, the difficulties encountered to date underscore the importance and urgency of investigating the mediation systems of inflammatory injury. In the mediation systems one finds many features common to various inflammatory diseases, such as the participation of complement components, leukocytes and effector molecules. Knowledge of one system, may well apply to the mediation of a second. And through analytic studies, the members of the mediating pathways become characterized and thereby vulnerable to specific pharmacologic attack. Thus the pharmacologic inhibition of the third component of complement, the prevention of release of lysosomal constituents of neutrophils to the surrounding milieu or the inhibition of released leukocytic protease may signal a new era of specific therapy.

This laboratory has been engaged for a number of years in the analysis of several of the mediating sequences. A portion of this effort has been supported by the Council for Tobacco Research. We propose now to further these investigations and in particular, to apply a number of findings from in vitro studies to models of disease in vivo. It is our contention that such studies are essential to the understanding of inflammatory injury of the lung brought about by any inciting agent. And such studies will hopefully lead to rational therapy of inflammatory disease of the respiratory system.

1. Studies of the fibrinolytic, intrinsic clotting and kinin forming systems in inflammatory injury.

In our previous studies, the initial members of these systems in rabbit and human plasma have been isolated, purified and characterized. Antibodies have been produced to each. A summary of progress has been appended (Appendix A) and the sequence of action tabulated (Table I and Fig. 1). The activation of the initial component, Hageman factor, follows binding to various membranes such as vascular basement membranes or collagen bundles, or is caused by enzymatic action of kallikrein, plasmin and clotting factor XI (listed in order of potency). The marked enzymatic activation in fluid phase by kallikrein was a surprising finding and may well represent the major mechanism by which Hageman factor is activated in vivo (See Appendix A).

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8. Details of Experimental Design and Procedures: (Continued) - 3

a. Activation of Hageman factor on membrane surfaces.

In order to gain a better understanding of the surface activation of Hageman factor on vascular structures a series of experiments will be conducted to detect binding and activation of Hageman factor in vivo along vascular and extravascular membranes. Preliminary data from this and other laboratories have revealed that Hageman factor does bind and is activated by vascular basement membranes and collagen. We now have produced strong monospecific antibody to rabbit and human Hageman factor that have been conjugated with fluorescein. This will be employed to detect the exact position in sections of lung where Hageman factor binds. In addition, the binding of Hageman factor will be examined in acute inflammatory pneumonitis produced by antibody directed to rabbit pulmonary membranes. Hageman factor bound in diseased human lung will also be sought in experiments to be performed with Dr. Averill Liebow of the University of California, San Diego. Early studies will be directed toward acute and chronic inflammatory processes.

Investigation will also be conducted into the structure of extracellular membranes responsible for the binding and activation of Hageman factor. Extracellular membranes will be obtained from several sources: pulmonary alveolar membranes, collagen, vascular basement membrane (glomerular), basement membrane glycoprotein secreted by a parietal yolk sac carcinoma that has been adapted to tissue culture by Dr. Lewis Johnson (who has kindly provided the purified glycoprotein). In addition, a series of synthetic peptides bearing triplet amino acid residues common to groups of amino acid residues in collagen have been obtained from Dr. Darwin Prokop of Rutgers University. These peptides and several analogues will be employed in blocking experiments (they do not activate Hageman factor) designed to inhibit interaction of Hageman factor and the various membrane structures.

These extracellular membranes will be treated with various agents in order to solubilize peptide moieties. The soluble products will then be tested for binding and activation of Hageman factor or for inhibition of this process by isolated vascular basement membrane (prepared in highly purified form from rabbit and human glomeruli in this laboratory). Among the agents to be used for solubilization will be purified specific collagenase, trypsin and cyanogen bromide. Considerable experience in isolating soluble fragments of extracellular membranes has been gained in this department in the past several years.

These studies should provide considerable information on the position in normal and diseased pulmonary tissue where Hageman factor may be activated, and the structure of the membrane responsible for binding and activation. The information will provide a foundation upon which a greater understanding of the participation of the intrinsic clotting, fibrinolytic and kinin forming systems participate in pulmonary disease. The fact cannot be stated too strongly that essentially no firm information exists today regarding to the importance of these systems in inflammatory disease despite the immense potentiality of products of these systems to induce injury.

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8. Details of Experimental Design and Procedures: (Continued) - 4

b. The effect of inhibitors of the Hageman factor activated systems on the development of inflammatory disease. Employing the model of pulmonary and glomerular inflammation induced by heterologous antibody to the respective membranes, various inhibitors of the kinin forming, intrinsic clotting and fibrinolytic systems will be tested for their ability to inhibit development of disease. Assays of inflammatory injury will include histologic damage to cells and membranes, deposition of fibrin, accumulation of leukocytes and intravenously injected colloidal carbon along vascular membranes indicating increased vascular permeability. In the case of glomerular injury, proteinuria will be measured quantitatively. In each case, the antibody will be labelled with ^{125}I and its binding in the tissues measured (using ^{131}I normal globulin as a paired-label control) in order to control the quantity of inciting agent participating in the reaction.

Inhibition will be conducted by mediation systems. It is known that both pulmonary and glomerular injury have two mediation pathways, one requiring complement and neutrophils, and another producing injury in the absence of these. We have found previously that the neutrophil-complement independent injury of glomeruli is qualitatively different from the injury resulting from the participation of these mediators in experimental glomerulo-nephritis. Thus inhibition experiments will be conducted in both the absence and presence of neutrophils. Pulmonary and glomerular inflammation will be produced by injection of ^{125}I antibody so that the quantity of bound antibody can be assessed. Inhibitors will then be tested in various groups of rabbits: the fibrinolytic pathway will be blocked by injection of epsilon aminocaproic acid or Mercke compound 576 which prevent activation of plasmin. Fibrin will form along glomerular and pulmonary vascular membranes as we have observed by both light and electron microscopy. The amount of fibrin should be much greater in treated animals. However with the inhibition of plasmin generation, a corresponding decrease in the rate and quantity of formation of fibrin split products will be observed. The presence of split products will be monitored by injecting ^{131}I rabbit fibrinogen with detection of labelled peptides in the urine and plasma. Split products in plasma will also be measured by their capacity to bind to and precipitate polyanions and plasmin generation at the site of injury assessed by the micro-fibrinolytic assay with frozen sections of tissue. The assay of plasmin activity is not quantitative, but will allow us to determine if fibrinolytic (plasmin) activity is present.

These studies will provide evidence on the importance of plasmin formation in the development of injury in both normal and neutrophil depleted animals, and indirectly of the role of fibrin split products. Control studies will be conducted on possible alternative effects of EACA and Mercke compound #576: inhibition of activation of the complement system, immune adherence of neutrophils, release of neutrophilic enzymes, inhibition of the enzymes themselves, the generation of Hageman factor activity and members of the three systems activated by this component, and finally, inhibition of platelet and monocytic functions. These control assays are routinely performed in this laboratory.

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8. Details of Experimental Design and Procedures: (Continued) - 5

c. The role of fibrin formation, and indirectly of its split products in the generation of pulmonary and glomerular lesions will be examined by depleting rabbits and rats of fibrinogen prior to injection of ^{125}I antibody. Two fibrinogenolytic enzymes, Venacil and Reptilase, derived from snake venoms will be employed, each of which exhibits marked specificity for fibrinogen. In our experiments, these enzymes can be injected safely 10-24 hours before an experiment resulting in marked depletion of fibrinogen but without measurable effect on the various systems noted under part b in the previous paragraph. Fibrinogen levels will be followed by use of thrombin-induced clotting and immunodiffusion assays.

d. The mechanism of neutrophil independent injury of pulmonary and glomerular membranes will also be studied by inhibition with the polypeptides Trasylol and pepstatin, and the inhibitors of trypsin derived from lima beans, soybeans and ovomucoid. These inhibitors are not specific to individual enzymes, but considerable information is being derived in this and other laboratories as to the enzymes in various mediation systems that are inhibited. This information is now being completed in the complement, fibrinolytic, kinin-forming and intrinsic clotting systems. A portion of these studies have been published (Appendix A). Their action on various leukocyte-derived enzymes is now being assessed. Each can be infused into experimental animals and the inhibitory effect on inflammatory lesions evaluated. In addition, several new enzyme inhibitors are available for evaluation, each of which inhibits kallikrein.

2. Studies of inflammatory injury mediated by leukocytes and other cells.

a. Neutrophils

We have recently devised a method of isolating and purifying peripheral neutrophils and infusing them into animals depleted of neutrophils. If the animals are prepared with antibody or immune complexes deposited along vascular membranes, the infused neutrophils reconstitute the reaction. We are now in a position to assess various functions of neutrophils in the development of inflammatory injury. The isolated neutrophils will be treated so as to block various functions thought to be important in their injurious capacity. They will then be infused into neutrophil depleted rabbits prepared with ^{125}I antibody which is bound to pulmonary or glomerular membranes. Injury will then be assessed as noted above. Comparison of the effect of inhibited neutrophils will be made with that of normal neutrophils and with animals in which neutrophil reconstitution is not made.

Several functions of neutrophils will be tested in this manner:

1) Immune adherence will be blocked by exposure of the neutrophils to freshly activated C3. The C3b will bind to the immune adherence reactive site on the neutrophil as determined by isotope marker on the C3b. The cells will be tested for their adherence capacity in vitro and for their ability to bind in test rabbits to pulmonary vascular membranes to which antibody and complement components are fixed. Adherence will be determined histologically.

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8. Details of Experimental Design and Procedures: (Continued) - 6

As a corollary to the above, the time of decay of membrane-fixed C3b will be assessed by infusing neutrophils at various times into neutrophil-depleted rabbits, injected with anti-lung membrane or anti-GBM antibody. C3b, which binds rapidly after injection of antibody, is degraded by an inhibitory enzyme in plasma, the so-called conglutinin-activating factor (KAF). In preliminary tests, when neutrophils were replaced 24 hours after the injection of antibody, the cells did not adhere to the antibody and complement along vascular membranes. This indicated the complement had been altered during this time so as not to bind neutrophils. As control, cutaneous Arthus reactions will be applied at various times to insure that neutrophils are capable of binding to fresh antigen-antibody-complement sites.

These results will determine for the first time the decay rate of bound and activated complement components involved in the accumulation of neutrophils in vivo.

2) Inhibition of release of lysosomal constituents will be accomplished by treating the isolated neutrophils with PGE₁, methyl xanthines or iodoacetate. Each of these has been found in this and other laboratories to inhibit the release of lysosomes to the exterior upon stimulation of the cell with antigen-antibody complexes (Appendix A). The effect of these inhibitors will be confined to the neutrophils since the animal will not receive the inhibitor. Side effects will thereby be averted, and specificity of inhibition will be maintained.

These studies are of great importance in view of the role of neutrophils in tissue injury. And since α -1-antitrypsin is a strong inhibitor of neutrophilic protease (elastase) in man, these enzymes are strong candidates for the injury and resulting fibrosis of lung in α -1-antitrypsin deficiency states. In this regard, the studies noted in part 1d above on the inhibition of inflammation with pepstatin are of importance. We have recently observed that pepstatin inhibits human neutrophilic proteases.

b. Macrophages, mast cells and platelets. Studies have been conducted in this laboratory over the past three years on the involvement of alveolar macrophages, mast cells, basophils and platelets in tissue injury, and the mechanisms of release of vasoactive amines and other constituents from these cells (Appendix A). The biochemical basis for this release process will be further studied including an examination of the role of cyclic AMP (here apparently an important modulator of release), serine esterases and microtubules and microfilaments.

These studies will be performed with the aid of various inhibitors of microtubular formation (colchicine and vinblastine), microfilaments (cytochalasin B) and factors that affect levels of cAMP (β and α activating and inhibiting agents, methyl xanthines). The inhibitors will be applied to cells stimulated with aggregated immunoglobulins, and active moieties of the complement system. The techniques involved are in common use in this laboratory.

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8. Details of Experimental Design and Procedures: (Continued) - 7

The platelet activating factor (PAF) (Appendix A) which we have found to be released from basophils will be further analyzed. PAF has been shown now to cause clumping of platelets with release of their vasoactive constituents and to induce an increase in vascular permeability. Recent preliminary studies have revealed that PAF is released from isolated, sensitized rabbit lung upon addition of antigen or anti rabbit IgE. This represents a new mediator of inflammation in lung the role of which must be determined. We now plan to study the mechanisms of its release from lung, immunoglobulin classes so involved and the means by which control of the release may be excercized. The cell of origin in the lung will be sought by desensitizing mast cells with anti IgE before adding antigen and vice-versa.

c. Interrelation between these systems. In the inflammatory reaction many of the humoral and cellular mediating processes are interrelated. Histamine can inhibit release from other cells and will be studied for its inhibition of neutrophil and platelet release. This could exert a controlling influence in vivo. Neutrophil and platelet accumulation is often accompanied by fibrin deposition as is seen in pulmonary lesions. Study of the connection between neutrophil release and initiation of coagulation will be undertaken. The affect of neutrophil enzymes and constituents on Hageman factor will be investigated along with the alternative possibility that platelets and neutrophils can be activated by components of the coagulation and kinin systems, just as they are by components of the complement system.

Summary of the relationship of these studies to pulmonary disease.

These studies will provide considerable understanding of the mechanisms of injury of the lung.

1. The importance of the intrinsic clotting system, platelets and neutrophils in the deposition of fibrin and injury of structure in acute pneumonitis will be assessed.
2. The increased vascular permeability in lung mediated by the kinin forming system and fibrin split products will be assessed.
3. The potential role of the platelet activating factor (PAF) in the development of pulmonary injury will be evaluated.
4. The mechanisms of release of injurious constituents of cells commonly found in pulmonary inflammation will be analyzed. Control mechanisms of the release process and inhibitors of the released injurious constituents will be examined.

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Appendix A

Report of Progress 1970-73

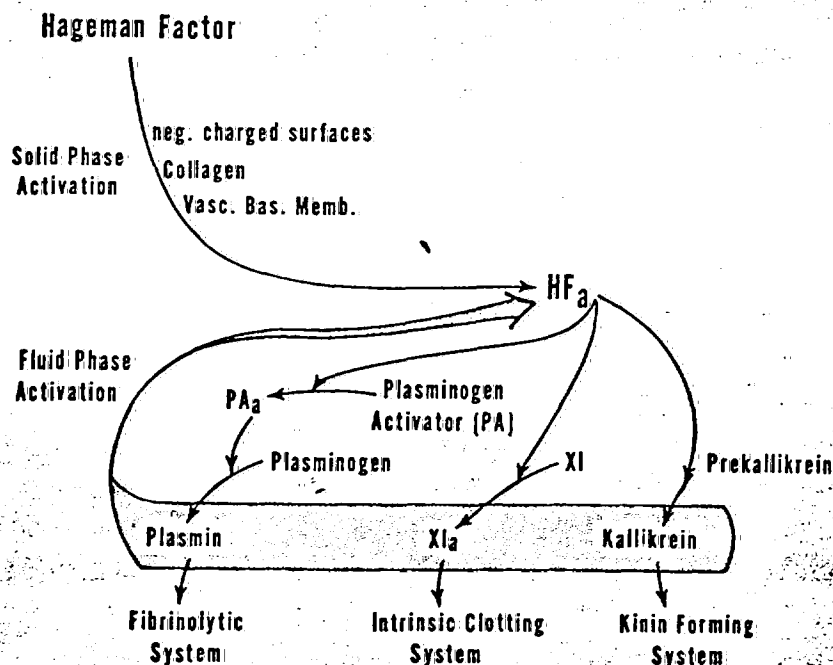
1) The kinin forming and intrinsic clotting systems.

a) Purification and characterization.

In the past three years, in this laboratory, each component of the kinin forming system has been isolated and purified in both human and rabbit plasma. In addition, Factor XI of the intrinsic clotting system has been purified and characterized. This has been accomplished by a combination of precipitation in ammonium sulfate, anion exchange chromatography, gel filtration and block electrophoresis (1-7). In each case, purity has been determined by the appearance of single protein bands in polyacrylamide electrophoresis. Of greatest importance, the molecules have been obtained in inactive, precursor form. This has allowed studies to be performed on the mechanisms of activation of each component as will be noted below. The information obtained in these and future studies will form the basis of an eventual understanding of the role of the kinin forming and intrinsic clotting systems in disease. The point must be underscored that unequivocal evidence of the participation of the kinin forming and intrinsic clotting systems in health and disease are lacking. The reason behind this void of information lies in the unavailability until now of purified precursor components of these systems.

The following figure and table illustrate several characteristics of the precursor components and their interaction as determined in this laboratory:

Figure 1



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Table 1

Physical Properties of the Components of the Hageman Factor Activated Systems in Human and Rabbit Plasma

	Hageman factor	Activated Hageman Factor (prekallikrein activator PKA)	Prekallikrein	Kininogen	Clotting Factor XI PTA	Plasminogen activator	Plasminogen
<u>Human</u>							
Molecular Wt.	90,000	30,000	107,000	70,000	160,000	100,000	80,000
Sed. Rate	4.5	2.6	5.1	3.8	7		
Electroph. mobility	beta	pre-albumin	gamma	alpha	gamma	gamma	beta
Isoelectric pt.	6.1	4.6			>7.5	8.7-9.0	6.3-8.6
<u>Rabbit</u>							
Molecular Wt.	90,000	30,000	99,000	79,000			
Sed. Rate	4.5	2.6	4.5	3.8			
Electroph. mobility	beta	pre-albumin	gamma	alpha			
Isoelectric pt.	6.1	4.6	5.9	5.2			

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b) Activation of individual components.

1. Hageman factor. Studies of the activation of Hageman factor have been aided by the ability to label the molecule with ^{125}I . This has allowed us to determine that molecular cleavage of the parent molecule has taken place. A variety of proteolytic enzymes have now been found to activate Hageman factor: trypsin, kallikrein and plasmin. The parent molecule upon activation by trypsin cleaves into three subunits of identical size, 30,000 daltons (8, 9). The daughter molecules possess the capacity to activate purified prekallikrein and clotting factor XI and have been termed prekallikrein activator (PKA). In addition, upon reduction with 0.02 M dithiothreitol, ^{125}I labelled Hageman factor falls into three subunits of equal charge and size 30,000 daltons. This indicates that Hageman factor consists of three equal polypeptide chains held together by disulfide bonds at the far end of the polypeptide chains (since trypsin cleaves the parent molecule similarly).

Among the non-enzymatic activators, collagen and glomerular basement membrane have been found to induce activation of Hageman factor. This has been revealed by binding of ^{125}I Hageman factor to these insoluble membranes and by its subsequent activation of purified prekallikrein or clotting factor XI. By contrast, human immunoglobulin of each class and subclass upon aggregation fail to activate Hageman factor. Antigen-antibody complexes prepared in many different ways also failed to activate as well as rheumatoid factors after combination with aggregated γ globulin. We have thus gained strong evidence against immunologic activation of this important factor.

Enzymatic activation of Hageman factor has been established. Kallikrein was found to activate Hageman factor in fluid phase with great sensitivity. Plasmin and Factor XI were found to activate but required 5 and 10 fold greater amounts respectively. The fluid phase activation gained importance when it was found that less than 1% of available Hageman factor binds to and is activated by the potent surface activator kaolin in the presence of undiluted plasma. Kallikrein has been found to promote clotting of plasma (10), in part, at least, through the activation of Hageman factor. The importance of kallikrein in activation of Hageman factor and the three systems was determined by studies of plasma exhibiting the Fletcher Factor deficiency. When exposed to glass, this plasma fails to form kinin, plasmin and clots with a greatly increased time. We determined that the missing factor was actually prekallikrein by functional and immunochemical means (11). Addition of prekallikrein restored the clotting, plasmin and kinin forming capacities.

PKA possesses enzymatic activity, being capable of splitting substituted esters of lysine (AGLMe, TLMe, CBZLMe, ALMe) but not esters of arginine (BAEe, BAME, BAPN, AAMe). Analysis of the initial rate of activation of prekallikrein by PKA supports the enzymatic nature of the reaction. In addition, lima bean trypsin inhibitor, DFP and phenyl-methylsulfonyl-fluoride block the activity of PKA.

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Antibody has been prepared to human Hageman factor, and quantitative immune diffusion analyses conducted. Initial studies reveal that normal human plasma contains 165 μg Hageman factor/ml. Fluorescein labeling of the antibody has been successfully performed. We are now in a position to analyze quantitatively for the first time the levels of Hageman factor in plasma and fluids in various disease states, turnover times of the ^{125}I labelled molecule and the binding of Hageman factor at sites of injury.

2. Prekallikrein. PKA cleaves rabbit prekallikrein into two fragments of 86,000 and 11,000 daltons (6). The larger of the two possesses kallikrein activities (arginine esterase and kinin release from kininogen). Human kallikrein is also cleaved, but reduction of the molecule is required to demonstrate that cleavage has occurred.

3. Factor XI of the intrinsic clotting system in human plasma has been isolated and purified. This molecule of 160,000 daltons consists of two polypeptide chains of equal size joined by disulfide bonds. Antibody to Factor XI has been prepared. Upon activation by PKA, Factor XI hydrolyzes synthetic amino acid substrates of arginine and lysine and is inhibited by DFP (12).

2) Neutrophil-mediated immunologic injury.

One of the in vivo models which will be employed in this study is the experimental arthritis which has been developed in this laboratory (13). This comprises a reversed passive Arthus reaction which is initiated in the rabbit stifle joint. The injury can be quantitated and requires complement and neutrophils. Most importantly, by replacing neutrophils into the joint of a neutrophil-depleted rabbit, the injury has been restored (13, 14). This reaction requires the migration of the neutrophils from joint space to the site of immune complex formation in the blood vessel walls. The migration was shown to be dependent upon C3 and C6 and is the first clear indication in vivo of the role of complement-derived chemotactic factors. Once the neutrophils have reached the complexes, phagocytosis occurs and it is presumed that tissue injury again follows the release of lysosomal constituents from the neutrophils.

More recently, experiments have indicated that it is also possible to re-establish lesions of nephrotoxic nephritis in neutrophil-depleted rabbits by intravenous injections of neutrophils. The neutrophils became bound in glomeruli only when rabbits were pretreated with nephrotoxic antibody (selected to produce injury in the presence of neutrophils). The neutrophils became bound in their first pass through the glomeruli. Injury to the glomerular basement membrane and proteinuria rapidly ensued. In other studies, three different types of antibody were obtained from fractions of sheep anti-rabbit glomerular basement membrane antiserum. One of these induced the neutrophil-dependent glomerulonephritis which will be used in these replacement studies, another produced a neutrophil-independent injury the pathogenesis of which is currently under investigation, and the third became bound to the basement membrane but induced no damage at all (15, 16).

1003538524

Release of granule enzymes from neutrophils phagocytosing immune complexes and particles has been demonstrated by a number of workers. The mechanism of release in vitro is being studied in this laboratory and has already been shown to follow degranulation into a phagocytic vacuole which may be open, or may later become open, to the exterior (17, 18, 19).

In addition, study of neutrophils in vitro (17, 18, 19) has revealed that granule enzymes of a potentially injurious nature are released from the cells when they are adherent to antibody or complement bound to non-phagocytosable surfaces (like the basement membranes). The in vitro observation showed that in the system employed, direct exocytosis of the neutrophil granules was the mechanism of release. It now becomes important to demonstrate this in vivo. Studies of these reactions in vitro are continuing (16, 20) and are beginning to define the biochemical reactions of the cells which culminate in exocytosis of granules ("degranulation").

The release is inhibited by agents which increase intracellular cyclic AMP (prostaglandins, phosphodiesterase inhibitors), which inhibit glycolysis, or by diisopropyl fluorophosphate which inhibits serine esterases. Calcium is also required for the release. These phenomena with neutrophils closely resemble release mechanisms of other cells.

Continuing studies on the mechanisms of deposition of circulating immune complexes have revealed that the increased vascular permeability, which is essential for deposition of the circulating complexes in vessel walls, may result from the interaction of sensitized leukocytes with antigen and platelets. In the reaction, the platelets clump and release vasoactive amines (20, 22). The leukocyte involved has now been shown to be a basophil and the reaction may be transferred to leukocytes of a normal rabbit with IgE antibody (22, 23, 24). Using cells from human beings sensitive to various antigens, data are emerging that indicate a similar reaction system (25). A soluble factor (PAF) was found to be released from the sensitized basophils and this mediated the clumping of platelets and release of their vasoactive amines (24, 26). This factor has been shown to bind readily to albumin, and to be extractible with ethyl alcohol (22, 25).

1003538525

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1003538526

902 CALISTON

1003538527

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 2, 1973

Grant Application No. 902

To: The committee comprising Drs. Jacobson, Loosli and Sommers

Subject: Morton Galdston, M.D., New York University Medical Center,
New York, N. Y.

New application No. 902

"Protease-Antiprotease Determinants: Role in Etiology and
Pathogenesis of Chronic Obstructive Pulmonary Disease"

History

The applicant telephoned Dr. Hockett, who did not encourage application. Nevertheless, Galdston stated he would submit because the application was "all ready".

Subsequent inquiry revealed that ^{this} is essentially a duplication of an NIH application. Further inquiry revealed that the status of that application is "recommended but not funded".

The request is for \$43,492 plus two additional years.

Documents Submitted

At this time we are forwarding only the application dated January 29, 1973 (19 pages).

Voluminous reprints, manuscripts and abstracts were also provided. These are not forwarded at this time for reasons apparent below.

Comment

Inquiry to Dr. Alice Berger, named as Statistical Consultant revealed that she:

1. Has not made any time commitment for this study.
2. Did not know she was included in this application.
3. Is not sure that she would be "allowed to participate in a study funded by CTR".

In view of the above, and since Dr. Hockett points out that we are now supporting investigations of this topic, perhaps action may be, at the most, deferral for a site visit.

FWN:wg
Encl.

F.W.N.

1003538528

CHRONIC PULMONARY DISEASES

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

Comm. 110 EAST 59TH STREET
Dr. Jacobson NEW YORK, N. Y. 10022
Dr. Loosli (212) 421-8883
Dr. Soumiers

Application for Research Grant
(Use extra pages as needed)

Date: 1/29/73

1. Principal Investigator (give title and degrees):

Morton Galdston, M. D., Associate Professor of Medicine

2. Institution & address:

New York University Medical Center
550 First Avenue
New York, N. Y. 10016

3. Department(s) where research will be done or collaboration provided.

Department of Medicine

4. Short title of study:

Protease-Antiprotease Determinants: Role in Etiology and Pathogenesis of Chronic
Obstructive Pulmonary Disease

5. Proposed starting date: 7/1/73

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

Our recent studies suggest the predilection of individuals deficient in alpha₁-antitrypsin (A₁AT) to develop chronic obstructive pulmonary disease (COPD) may be partly determined by excess polymorphonuclear leukocyte (PMN) lysosomal neutral protease activity relative to serum antiprotease activity (1,2). These findings and the recent discovery of an antiprotease secreted by ciliated bronchial epithelial cells (3,4) have stimulated us to plan expansion of our investigations of the role of derangements of circulating and local protective mechanisms of lung in the etiology and pathogenesis of COPD, ultimately to devise ways to detect those likely to develop it and methods for prevention and treatment.

Our specific research aims are:

1. to elucidate the role in etiology and pathogenesis of COPD of:

- a. an imbalance between
 - (1) serum antiprotease and peripheral leukocyte lysosomal protease activity
 - (2) antiprotease and protease activity in bronchial secretions
- b. inflammation in accentuating or inducing these imbalances

2. to investigate the effect of smoking on:

- a. protease-antiprotease balance in peripheral leukocyte lysosomal protease and serum antiprotease activities
- b. protease-antiprotease balance in bronchial secretions
- c. lysosomal protease activity of alveolar macrophages.

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2. Brief statement of working hypothesis.

Though individuals with severe A_1AT deficiency (ZZ homozygotes) have a strong predilection to develop COPD at a relatively young age, some do not, even when elderly (5,6). It is controversial whether COPD also occurs with greater frequency among those with moderate A_1AT deficiency, heterozygotes (7,8). Cigarette smoking probably enhances the likelihood in such individuals (9) as it does in those with normal A_1AT (10).

The level of A_1AT may be only one among many determinants of COPD, perhaps genetic, environmental (particularly cigarette smoke) and local protective mechanisms of lung. Whether COPD develops with or without A_1AT deficiency, its rate of progression and pattern of expression could depend upon the interplay and cumulative effects of such factors.

Supportive evidence for the role of lysosomal protease in the etiology and pathogenesis of COPD comes from several experimental observations: human leukocyte lysosomal protease can degrade constituents of lung tissue such as basement membrane (11,12), arterial wall (11,12), collagen (13) and protein polysaccharides of cartilage (14,15); structural changes consistent with human emphysema have been induced in dog lung with aerosolized homogenates of dog and human polymorphonuclear leukocytes and of dog alveolar macrophages of known proteolytic activity (16);

9. Details of experimental design and procedures (append extra pages as necessary)

(cont'd pg. 3)

Subjects

We will investigate the following groups of subjects:

1. those with COPD
 - a. homozygous deficient for A_1AT
 - b. heterozygous deficient for A_1AT
 - c. homozygous for the normal A_1AT gene
2. normals free of lung disease
3. family members of probands deficient in A_1AT
4. individuals undergoing bronchoscopy or with endotracheal or tracheostomy tube in place
5. individuals undergoing lung resection.

The subjects will be volunteers gathered from the following sources:

1. Emphysema Clinic of the Chest Service, Bellevue Hospital, which annually has a census of approximately 200 patients, with about 30 to 40 deaths and an autopsy rate of 25 to 50 per cent, screens about 100 referrals, of which about 50 are added to the Clinic census.
2. patients with COPD admitted to the three hospitals of New York University Medical Center: Bellevue, University and Manhattan Veterans'
3. referrals from attending physicians and from the community
4. family members of those found deficient in A_1AT
5. healthy individuals and patients without lung disease.

We will base the diagnosis of COPD on history, physical findings, chest roentgenogram, electrocardiogram and pulmonary function tests.

Pulmonary function tests will include total lung capacity by the helium dilution method, ratio of residual volume and of functional residual capacity to total lung capacity, arterial pO_2 , pCO_2 , pH, plasma $BHCO_3^-$ and steady state carbon monoxide diffusing capacity, forced vital capacity (FVC), percentage expired in one second ($FEV_1\%$), forced mid-expiratory flow ($FEF_{25-75\%}$) and forced expiratory flow ($FEF_{200-1200}$).

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8. Brief statement of working hypothesis (continued)

purulent sputum contains leukocyte proteases capable of degrading lung tissue; these proteases are inhibited by A_1AT and only to a limited degree by A_1AT -deficient serum (17), which also has a limited capacity to inhibit elastase (18,19) and collagenase (19).

Lung tissue may be particularly vulnerable to such proteases because it has the largest vascular bed of any organ, receives the entire cardiac output, and the potential number of sequestered leukocytes and of alveolar macrophages releasing proteases into the extravascular space is greater than in other organs.

A plausible explanation for the failure of some individuals with A_1AT deficiency to develop COPD might be that they have a concomittant low PMN lysosomal protease activity sufficient to maintain a normal protease-antiprotease balance, whereas those developing lung disease, whether homozygous or heterozygous deficient, or with normal A_1AT , would have normal or increased PMN protease activity and therefore excess protease relative to antiprotease (A_1AT) activity. Such protease-antiprotease imbalance would be greater in homozygotes than in heterozygotes which could account for the greater predilection of the former to develop COPD and at an earlier age (20).

Although there is a history of cigarette smoking in virtually all patients with well-documented COPD (10), only approximately 50% of individuals with a comparable history of long-standing heavy smoking exhibit moderate to advanced emphysema at post mortem (21), again indicating the probable role of several determinants.

The greater predilection of smokers than non-smokers for COPD could be related to a variety of factors tending to induce a less favorable protease-antiprotease balance. Cigarette smokers, particularly those who inhale, have a higher circulating white blood cell count without change in the differential count (22) and therefore are prone to sequester a greater number of PMN in lung. They thus have a potentially richer source for release of lysosomal protease in lung. Also, lung wash of smokers has a greater number of alveolar macrophages, another source for local release of lysosomal protease, and less surfactant (23). The latter suggests one of the local defense mechanisms of lung, surfactant, may be compromised in smokers, since we recently found it to be a potent inhibitor of non-antigenic bacterial cytolytic toxins capable of releasing lysosomal proteases from PMN and alveolar macrophages (24). In addition, pathologic changes in bronchial epithelium of cigarette smokers could alter the local protease-antiprotease balance of lung by inducing enzymes in leukocytes and in alveolar macrophages and by altering the quantity or protective properties of the low molecular weight antiprotease secreted by ciliated bronchial epithelium (3,4) or the rate of diffusion of A_1AT from blood into bronchial secretions (25), without an accompanying change in protease-antiprotease balance in circulating blood.

The low molecular weight antiprotease of bronchial secretions has a similar protease inhibitor spectrum as A_1AT and normally accounts for about 70 per cent of the antiprotease activity of bronchial secretions; the remainder results from the presence of A_1AT (3).

Thus, by investigating protease-antiprotease balance in bronchial secretions along with that in the peripheral blood, under a variety of clinical conditions, we should be able to delineate factors (including cigarette smoking) determining these balances and their role in the etiology and pathogenesis of COPD.

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9. Details of experimental design and procedures (continued):

Blood Samples

Fifty cc of peripheral venous blood will be drawn; 2 cc will be used for total blood cell count by a Coulter counter, microhematocrit and hemoglobin determinations, and differential white blood cell count. Eight cc of blood will be used to prepare serum for detecting abnormalities of A₁AT and the remaining 40 cc will be processed to prepare leukocyte lysosomal extract according to Janoff and Scherer (26).

Methods for Detecting Alpha₁-Antitrypsin Abnormalities

We will measure serum total trypsin inhibitory capacity (mg trypsin inhibited per ml serum), an index of the total antiprotease activity of serum, according to Eriksson (5). This correlates well with the level of serum A₁AT measured by radial gel immunoassay (27) and by electroimmunodiffusion (20). Values for TIC fall into three groups: normal, 0.80 to 1.40 mg/ml; intermediate low or heterozygous, 0.40 to 0.79 mg/ml; and low or homozygous deficient, less than 0.40 mg/ml (5).

We will perform phenotyping, proteinase inhibitor (Pi), by acid starch gel electrophoresis and crossed electroimmunodiffusion, according to Fagerhol (28). This is a reliable method for detecting heterozygotes with borderline normal TIC and A₁AT, and heterozygotes and homozygotes whose TIC and A₁AT levels rise to normal and low heterozygous range, respectively, with inflammatory disease, estrogens and pregnancy (29,30).

We will use the electroimmunodiffusion method (31,32) to measure A₁AT. We will use a reference serum to be obtained from Dr. Richard C. Talamo, Massachusetts General Hospital, to check on methodology for serum TIC, phenotyping and electroimmunodiffusion and also to standardize a reference serum we will prepare from pooled specimens of normal subjects. Dr. Talamo has kindly consented to assist us in setting up the phenotyping and electroimmunodiffusion methods. Serum specimens will be divided among many small vials and stored at -80 C.

Leukocyte Lysosomal Enzyme Activity

Neutral protease activity of leukocytes has been demonstrated against a broad spectrum of substrates but it is not known how many neutral proteolytic enzymes contribute to tissue damage or to what extent they vary among different individuals. Thus far our most extensive experience has been in measuring two neutral protease activities: elastase-like esterase and leukoprotease. It seems likely that the elastase-like esterase plays an important but not exclusive role in PMN-mediated tissue damage (33). It has recently been found that leukoprotease activity is also partly a measure of elastolytic activity (34). Consistent with this is the highly significant coefficient of correlation, $r = 0.62$ ($P < 0.001$) (80 paired observations), we found between these two measurements (2).

It appears important to investigate whether variation in the level of other neutral leukocyte lysosomal protease activities in addition to those we have been measuring, may, in association with A₁AT, also be determinants of COPD. With this in mind we recently began to measure casein digestion activity.

Other recently reported neutral protease activities of leukocyte lysosomal extract to be considered are: the fibrinogen-splitting enzyme (35); the "labile" protease active on native protein, using the substrate native bovine hemoglobin (17); and the histone-degrading enzyme found in rabbit PMN (36).

1003538532

9. Details of experimental design and procedures (continued):

Though the role of leukocyte lysosomal acid proteases in tissue damage in vivo is yet to be clearly established it appears that under some conditions neutral and acid protease would act synergistically in extracellular spaces (37). We therefore have also recently begun to measure acid protease activity.

Methods for Measuring Enzyme Activities in Leukocyte Lysosomal Granule Extract:

1. elastase-like esterase with the substrate tertiary-butyloxycarbonyl (t-BOC)-l-alanine p-nitrophenyl ester, according to the method of Visser and Elout (38) as modified by Janoff (39) expressed as the amount of p-nitrophenyl liberated on hydrolysis of the ester $\Delta E_{347.5/30 \text{ sec}/25 \mu\text{g}}$ granule extract protein
2. leukoprotease (a guage of total neutral proteolytic activity) with denatured hemoglobin as substrate, according to Anson (40) as modified by Press, Porter and Cebra (41), expressed as TCA supernatants $\Delta E_{280/10 \text{ min}/25 \mu\text{g}}$ granule extract protein
3. casein digestion (42) with alpha-casein as substrate, expressed as μg protein digestion/30 min/25 μg granule extract protein, as modified by Dr. Aaron Janoff, Department of Pathology, State University of New York at Stony Brook (personal communication)
4. acid protease (pH 3.0) with denatured hemoglobin as substrate by the same method as leukoprotease (40,41) expressed as $\Delta E_{280/30 \text{ min}/50 \mu\text{g}}$ granule extract protein
5. beta-glucuronidase, a non-proteolytic leukocyte lysosomal enzyme, serving as a marker enzyme, according to Fishman, using phenolphthalein glucuronic acid as substrate (43), expressed as μg phenolphthalein formed per hour, per mg granule extract protein. Protein will be determined by the Lowry method (44).

Bronchial Secretions

Subjects

Bronchial secretions will be aspirated from healthy part of lung of individuals undergoing bronchoscopic examination, from patients with COPD, and others without lung disease who require an endotracheal or tracheostomy tube.

Annually about 100 patients are examined with the Olympus 5 mm fiberoptic bronchoscope on the Chest Service of Bellevue Hospital; about 250 to 300 undergo bronchoscopic examination on the Surgical and Ear, Nose and Throat Services of the New York University Medical Center; and, in addition, 80 to 90 lung resections are performed. Members of these staffs will collect bronchial secretions for our studies. Dr. Frederick Gorstein, pathologist, will permit us to study resected lung specimens.

We will review the smoking history, clinical course, and pulmonary function tests of those undergoing bronchoscopy or tracheal aspiration.

We will perform the following studies on these secretions:

1. bacterial culture
2. microscopic examination for purulence
3. protease inhibitory capacity by the total trypsin inhibitory capacity method (5), expressed as mg trypsin inhibited per mg protein in bronchial secretions.

1003538533

9. Details of experimental design and procedures (continued):

This is a measure of the available antiprotease activity in bronchial secretions, i.e., that which has not complexed with protease. Normally, 70 per cent of the antiprotease activity of bronchial secretions results from the low molecular weight antiprotease believed secreted by ciliated bronchial epithelial cells; the remainder is due to A₁AT which diffuses into lung from blood (3).

4. alpha₁-antitrypsin, by the electroimmunodiffusion method (31,32) using a modification to be published (see reference 11 in (45), obtained from Dr. Hyslop (personal communication). This will be expressed as A₁AT per mg protein in bronchial secretions.

5. total activity of the low molecular weight antiprotease of bronchial secretions. This is the activity of the free (uncomplexed component) and of the component complexed with protease. The latter is freed when bronchial secretions are processed according to Hochstrasser and co-workers (3,4). Perchloric acid (final concentration 5%) is added to secretions to precipitate higher molecular weight protein components, neutralized with KOH, forming a precipitate of KClO₄. Following centrifugation, the supernatant is removed, diluted with borate buffer, pH 8.0, 0.05 M, filtered and concentrated by pressure dialysis with intense stirring. An aliquot is used to measure trypsin inhibitory capacity (5). This will be expressed as mg trypsin inhibited per mg protein in bronchial secretions. Protein will be measured by the Lowry method (44).

6. protease activity, which may be due to lysosomal proteases of alveolar macrophages, leukocytes and bacteria, by the leukoprotease method previously described for leukocytes (40,41) and expressed in appropriate units per mg protein in bronchial secretions (40,41).

7. beta-glucuronidase activity, a measure of lysosomal enzyme release, by the method previously described for leukocytes (43) and expressed in appropriate units per mg protein in bronchial secretions (43).

We will collect a peripheral venous blood sample from the patients whose bronchial secretions are aspirated for studies of protease-antiprotease activity, as previously described, and total and differential white blood cell counts.

Alveolar Macrophages

We will lavage healthy part of resected lung with sterile saline solution according to Cohen and Cline to collect alveolar macrophages (31).

We will wash and resuspend in buffered saline (pH 7.0) the cell button sedimented from aspirated lavage fluid by centrifugation and remove an aliquot for total cell count, differential count using Giemsa stain, observation of cell types by phase-contrast microscopy and for dye-exclusion test with trypan blue (cell viability count).

We will prepare lysates of lysosomal granules of alveolar macrophages according to Janoff (47) and assay them for protease activity using methods already described. We will express enzyme activity per 25 μ g granule extract protein and also in relation to number of alveolar macrophages per ml.

It is possible that the enzyme activity we find in alveolar macrophages is derived from phagocytized leukocytes destroyed in lung. Studies carried out with Janoff tend to rule this out with regard to elastase-like esterase activity (47); it has yet to be excluded for leukoprotease activity of human alveolar macrophages. Regardless, lysosomal protease of alveolar macrophages would tend to destroy lung tissue in the presence of insufficient local antiprotease capacity.

1003538534

9. Details of experimental design and procedures (continued):

Analysis of Data

We will have the consultative assistance of an expert statistician, Dr. Agnes Berger, Associate Professor of Biostatistics, School of Public Health of the Faculty of Medicine, Columbia University, in analyzing our results.

We will analyze our data to learn whether:

1. there is a significant correlation among the various neutral protease activities and between each of them and acid protease activity
2. those who have developed COPD have an excess of leukocyte lysosomal protease activity relative to antiprotease activity (low or intermediate low TIC and normal or high protease)
3. those free of lung disease have a normal protease-antiprotease balance (low or intermediate low TIC and corresponding levels of protease activity)
4. those with COPD and the greatest protease-antiprotease imbalance have a more rapid downhill course than those with a lesser imbalance
5. smokers with a protease-antiprotease imbalance have an earlier onset and a more rapid progression of COPD than non-smokers with a comparable imbalance
6. leukocyte lysosomal protease activity is related to the number of mature or immature polymorphonuclear leukocytes
7. there is a population of individuals with COPD with normal TIC and abnormally high leukocyte protease activity
8. there is a pattern of familial transmission of the different leukocyte lysosomal protease activities. Since the enzyme specificity for these activities is not established, definitive conclusions concerning their possible genetic determination will have to await further investigation. Dr. Rody Cox, Section of Genetics, Department of Medicine, New York University Medical Center, will consult in these aspects.
9. the protease-antiprotease balance of bronchial secretions of smokers differs from non-smokers' and, if so, is it related to:
 - a. diminution of
 - (1) total content of low molecular weight antiprotease
 - (2) content of A_1AT
 - b. increase of protease activity from
 - (1) increase in number of alveolar macrophages and/or
 - (2) enhanced lysosomal protease activity

We will also seek to determine the effect of pulmonary infection and recovery therefrom on protease-antiprotease balance in bronchial secretions and in peripheral blood; and, similarly, the effect of non-pulmonary infection. Where possible these studies will be carried out in the same individual.

We will also perform serial measurements at intervals of about 6 months to a year in selected patients with COPD with low, intermediate low and normal serum TIC to investigate the relation of clinical progression of the disease with peripheral blood protease-antiprotease balance.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Sufficient space is available in the laboratory of the principal investigator, B5, Bellevue Hospital, to carry out all of the planned analytical procedures. In addition, a departmental cold room is available close by.

Among the major items of equipment are: table model high speed and clinical centrifuge, Beckman spectrophotometer, phase contrast microscope, pH meter, Mettler top-load and microbalances, temperature-control water bath, vacuum pump, incubator, sterile hood, flash evaporator, lyophilization apparatus and refrigerator with freeze compartment to -20 C.

Facilities to perform ventilatory pulmonary function measurements are available in the laboratory of the principal investigator. In addition, the facilities and services of the pulmonary function laboratory of the Chest Service, D2, Bellevue Hospital, under the supervision of Dr. Anne L. Davis, collaborator in this proposed research program, are available. The major items of equipment here are appropriate spirometers and a helium analyzer to measure total lung volume and subdivisions, dynamic airflow volumes, a blood gas tension analyzer and a pH meter and apparatus to measure carbon monoxide diffusing capacity.

11. Additional facilities required:

None

1003538536

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

12. Biographical sketches of investigator and other professional personnel:

Horton Galdston, M. D.

Role in Proposed Project: Principal Investigator

Title: Associate Professor of Medicine

Birthdate:

REDACTED

Place of Birth:

REDACTED

Education:

New York University, New York, N.Y., B.S., 1932

Columbia University, New York, N.Y., 1932-1933

New York University College of Medicine, New York, N.Y., M.D., 1937

Honors:

Alpha Omega Alpha

Brooks-Bowen Scholar in Medicine, New York Academy of Medicine

Army Commendation Ribbon for major contributions to lung irritant casualty treatment studies, establishment of objective criteria for evaluation of permanent lung damage by phosgene, development of oxygen therapy equipment

Office of Scientific Research Development certificate for participation in medical research in aviation medicine in World War II.

Research and Professional Experience:

1955-

1968-

1955-

1946-1955

1952-

REDACTED

1944-1946

1943-1944

1942-1957

1942-1943

1941-1942

1939-1941

1937-1939

Major Research Interest: Lung Disease, Pulmonary Physiology, Experimental Pathology

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10.

12. Biographical sketches of investigator and other professional personnel.(continued):

Morton Galdston, M. D. (continued)

Professional Societies:

REDACTED

REDACTED

1003538538

12. Biographical sketches of investigator and other professional personnel(continued):

Anne Logan Davis, M. D.

Role in Proposed Project: CollaboratorTitle: Associate Professor of Clinical MedicineBirthdate: RPlace of Birth: REducation:

Wellesley College, B. A., 1945

Columbia University College of Physicians and Surgeons, M. D., 1949

Intern, First (Columbia Univ.) Med. Div., Bellevue Hosp., 1949-1950

Asst. Res. & Res. Phys., Chest Service, Bellevue Hosp., 1954; 1955-1956

Resident Fellow (Amer. Trudeau Soc.), Chest Service, Bellevue Hosp., 1955-1958

Honors:

Alpha Omega Alpha, 1948

Research and Professional Experience:Hospital

REDACTED

1970-
 1963-1969
 1958-1968
 1/58-1963
 1954-1955
 1954-1955

University

Assoc. Prof. of Clinical Medicine, NYU School of Med.,
 Assist. Prof. in Med., College of P & S, Columbia Univ.
 Assoc. in Med., College of P & S, Columbia Univ.
 Instructor in Med., College of P & S, Columbia Univ.

1968-
 1964-1968
 1960-1964
 1957-1960

Major Research Interest: Pulmonary Disease

1003538539

12. Biographical sketches of investigator and other professional personnel (continued):

Dr. Agnes Berger

Role in Proposed Project: Statistical Consultant
(listed in #14. (First Year Budget) under C.)Biographical Information:

Associate Professor of Biostatistics, School of Public Health of the Faculty of Medicine, Columbia University, New York, N.Y. since 1964.

Ph. D. in Mathematics, University of Budapest, 1939.

R

Studied statistics with Neyman and Wald.

Publications of Medical Interest

Berger, A. and Gold, R.Z., On comparing survival times; Proceedings of the Fourth Berkeley Symposium on Mathematical Statistics and Probability held in 1960; Univ. of Calif. Press.

Gold, R.Z., Berman, S.H., Berger, A., On the question of whether a disease is familial; J. Amer. Statistical Assoc., 62: 409-420, 1967.

Additional Publications have appeared in the following:

Proceedings of the American Mathematical Society
Annals of Mathematical Statistics
Journal of the American Statistical Association

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13. Publications of Morton Galdston, M. D. (Principal Investigator):

1. Galdston, M. and Shah, D.O., Surface properties of dipalmitoyl lecithin in relation to lung surfactant, *Biochim. et Biophys. Acta*, 137: 235, 1967.
2. Galdston, M., Shah, D.O. and Shinowara, G.Y., Isolation and characterization of a lung lipoprotein surfactant, *J. Colloid and Interface Sci.*, 29: 139, 1969.
3. Galdston, M. and Shah, D.O., Lipid-protein association in lung surfactant In *Advances in Experimental Medicine and Biology: Surface Chemistry in Biological Systems*, Vol. 7, p. 261-274, Plenum Press, New York, 1970.
4. Janoff, A., Rosenberg, R. and Galdston, M., Elastase-like esteroprotease activity in human and rabbit alveolar macrophage granules, *Proc. Soc. Exper. Biol. Med.*, 136: 1054, 1971.
5. Galdston, M., Janoff, A. and Davis, A.L., Familial variation of leucocyte lysosomal protease and alpha₁-antitrypsin as determinants in chronic obstructive pulmonary disease. (Accepted for publication in *American Review of Respiratory Disease*)

In addition, two abstracts dealing with current research activities and referred to in the proposed grant application and submitted for meetings to be held in the spring of 1973 are appended. They are listed as bibliographic references #2 and #24.

1003538541

14.

14. First year budget:

A. Salaries (give names or state "to be recruited")
Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

Morton Galdston, M. D.

75

Anne L. Davis, M. D. (Collaborator)

Technical

Christine Gottwalt, Research Technician II

100

To be recruited " " "

100

10,261.

Employee Fringe Benefits (14% of salaries)

Sub-Total for A 24,358.

B. Consumable supplies (by major categories)

1. Reagents and Chemicals

1,000.

2. Enzymes

100.

3. Antiserum

500.

4. Glassware

75.

5. Disposable: Syringes and Needles

200.

Lambda and Pasteur Pipettes

125.

Storage Tubes

50.

Sub-Total for B 2,050.

C. Other expenses (itemize)

1. Statistics: Statistical Consultant (50 hrs. @ \$25/hr.)

1,250.

Key punching and Verification

750.

Computer Usage

300.

2. Maintenance of Equipment

500.

3. Publications, preparation of reports, manuscripts, charts, slides

500.

4. Books, Journals, Photocopying

100.

5. Travel to Meetings

350.

Sub-Total for C 3,750.Running Total of A + B + C 30,158.

D. Permanent equipment (itemize) *

1. Electrophoresis Equipment with Attachments

1,750.

2. Amicon Macrosolute Concentrating Apparatus with Accessories

500.

3. Lourdes Model 30-R Refrigerated Centrifuge

2,750.

High Speed Attachment

385.

Large Volume Rotor Head

585.

4. Revco Freeze Chest to -85 C

2,800.

*Please see page 15: Justification for Request

Sub-Total for D 8,810.

E. Indirect costs (15% of A+B+C)

E 4,524.Total request 43,492.

	Salaries	Consumable Suppl.	Other Expenses	Permanent Eq. p.	Indirect Costs	Total
Year 2	26,422.	2,550.	4,250.	0	4,983.	38,205.
Year 3	28,660.	3,050.	4,750.	0	5,469.	41,929.

1003538542

14 D. Permanent Equipment: Justification for Request

1. Electrophoresis equipment with attachments is essential for phenotyping and measurement of α_1 -antitrypsin content of serum and of bronchial secretions by electroimmunodiffusion. We do not own such equipment.

2. Amicon Macrosolute Concentrating Apparatus with accessories will permit us to separate and concentrate the low molecular weight antiprotease in bronchial secretions.

3. Lourdes Model 30-R refrigerated centrifuge with high speed attachment and large volume rotor head will permit us to handle efficiently the large volumes of lung wash and of blood we will have to process. We selected this model centrifuge because the rotor heads of our table model Lourdes centrifuge fit it. We currently are working under a considerable disadvantage because of the limited capacity of our table model centrifuge and the need to keep it in our refrigerator to attain a reasonably low temperature during centrifugation.

4. Freeze chest to -85 C. It is essential to store leukocyte and alveolar macrophage lysosomal extracts at -80 C to maintain their enzyme activity indefinitely. Facilities for long-term storage are essential for our contemplated research program because of the large number of extracts to be prepared, the practical necessity to analyze at one sitting several specimens prepared over a period of time, the need to maintain composite reference preparations and the desirability of having specimens available to explore new investigative leads. Loss of enzyme activity occurs in about 2 weeks at the lowest temperature attainable, -20 C, in the freeze compartment of our refrigerator.

1003538543

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Pulmonary Emphysema: Causes, Prevention and Treatment	Sundry private donors	Approx. \$10,000. per yr.	Not applicable

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Protease-antiprotease Determinants: Relation to COPD	N.I.H. HL 14700-01A1 PTHA	\$120,620.	7/1/73 - 6/30/76

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Concerning Conditions and Terms Under Which Project Grants Are Made."

Principal Investigator

Typed Name Morton Galdston, M. D.Signature Morton Galdston Date 1/29/73Telephone 212 OPECCN 9-3200 2681
Area Code Number Extension

Checks payable to

New York University Medical Center

Mailing address for checks

550 First Avenue
New York, N. Y. 10016

Responsible officer of institution

Typed Name PETER. ARAKELIANAssociate
Title DirectorSignature Peter Arakelian Date 1/2/73Telephone 212 OPECCN 9-3200 3977
Area Code Number Extension

1003538544

Bibliographic References

1. Galdston, M., Janoff, A. and Davis, A.L., Familial variation of leukocyte lysosomal protease and serum alpha₁-antitrypsin in chronic obstructive pulmonary disease. Accepted for publication, Am. Rev. Resp. Dis.
2. Galdston, M., Janoff, A., Gottwalt, C. and Davis, A. L., Leukocyte (PMN) lysosomal protease-alpha₁-antiprotease (A AT) imbalance in chronic obstructive pulmonary disease, (abstract) Submitted for the program of the National Tuberculosis and Respiratory Disease Association, 1973 Annual Meeting.
3. Hochstrasser, K., Reichert, R., Schwarz, S. and Werle, E., Isolierung und charakterisierung eines proteasen inhibitors aus menschlichem bronchialsekret, Hoppe-Seyler's Z. Physiol. Chem., 353: 221, 1972.
4. Reichert, R., Hochstrasser, K. and Werle, E., Nachweis eines niedermolekularen proteaseinhibitors in menschlichem bronchialsekret, Z. Laryng. Rhinol., 51: 190, 1972.
5. Eriksson, S., Studies in alpha₁-antitrypsin deficiency, Acta Med. Scand., 177: (Supp. 432) 1, 1965.
6. Fagerhol, M.K., Laurell, C.-B., The pi-system inherited variants in A.G. Steinberg and A.G. Bearn, Progress in Medical Genetics, Vol. VII, p. 107, Grune and Stratton, New York, 1970.
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8. Kueppers, F., Alpha₁-antitrypsin: physiology, genetics and pathology, Humangenetik, 11: 177, 1971.
9. Mittman, C., Lieberman, J., Marasso, F. and Miranda, A., Smoking and chronic obstructive lung disease in alpha₁-antitrypsin deficiency, Chest, 60, 214, 1971.
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11. Janoff, A., Zeligs, J.D., Vascular injury and lysis of basement membrane in vitro by neutral protease of human leucocytes, Sci., 161: 702, 1968.
12. Janoff, A., Mediators of tissue damage in leukocyte lysosomes, X. Further studies on human granulocyte elastase, Lab. Invest., 22: 228, 1970.
13. Lazarus, G.S., Daniels, J.R., Brown, R.S., Eladen, H.A. and Fullmer, H.M., Degradation of collagen by a human granulocyte collagenolytic system, J. Clin. Invest., 47: 2622, 1968.
14. Ziff, M., Gribetz, H.J. and Lospalluto, J., Effect of leukocyte and synovial membrane extracts on cartilage mucoprotein, J. Clin. Invest., 39: 405, 1960.
15. Janoff, A. and Elondin, J., Degradation of cartilage matrix by a neutral protease fraction of human leukocyte lysosomes, Proc. Soc. Exp. Biol. Med., 135: 302, 1970.

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16. Mass, B. Ikeda, T., Meranze, D.R., Weinbaum, G. and Kimbel, P., Induction of experimental emphysema, *Amer. Rev. Resp. Dis.*, 106: 384, 1972.
17. Lieberman, J. and Gawad, M.A., Inhibitors and activators of leukocytic proteases in purulent sputum, *J. Lab. Clin. Med.*, 77: 713, 1971.
18. Turino, G.M., Senior, R.W., Garg, P.D., Keller, S., Levi, M.M. and Mandal, I., Serum elastase inhibitor deficiency and α_1 -antitrypsin deficiency in patients with obstructive emphysema, *Science*, 165: 709, 1969.
19. Pierce, J.A., Eisen, A.Z., Dhingra, H.K., Relationship of anti-trypsin deficiency to the pathogenesis of emphysema, *Trans. Assoc. Amer. Phys.*, LXXXII: 87, 1969.
20. Mittman, C., Summary of symposium on pulmonary emphysema and proteolysis, *Amer. Rev. Resp. Dis.*, 105: 430, 1972.
21. Auerbach, O., Hammond, E.C., Garfinkel, L. and Penante, C., Relation of smoking and age to emphysema, *New Eng. J. Med.*, 286: 853, 1972.
22. Corre, F., Lellouch, J. and Schwartz, D., Smoking and leucocyte count, *Lancet*, 2: 632, 1971.
23. Finley, T.N. and Ladman, A.J., Low yield of pulmonary surfactant in cigarette smokers, *New Eng. J. Med.*, 286: 223, 1972.
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#899 GREENWOOD

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THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

3
January 30, 1973

Grant Application No. 899

To: The committee comprising Drs. Gardner, Loosli, and Meier

Subject: Martha F. Greenwood, M.D., University of Kentucky,
Lexington, Kentucky
New application No. 899
"A Study of the Functional Capacity of the Monocyte-
Macrophage System"

History

Dr. John H. Kreisher has discussed this proposal with Dr. Phillip Holland, the applicant's chief. The request is for \$24,035, plus two additional years.

Documents Submitted (attached)

1. Application dated 1/26/73.
2. Manuscript "Scanning Electron Microscopy . . ." by Greenwood and Holland.
3. Abstract "Bactericidal Capacity . . ." by Greenwood and others.

(Reprints of the three papers checked on the last page of the bibliography in the application were sent, and will be forwarded to you if you request).

Comment

Dr. Holland, Dr. Greenwood's chief and also co-investigator, states that if this request is granted, advice of CTR will be sought with regard to smoke exposure of animals.

At Dr. Kreisher's suggestion we are seeking outside evaluation from two CTR grantees who work in related fields.

F.W.N.
F.W.N.

FWN:wg
Encls.

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Comm.

Dr. Gardner
Dr. Loosli
Dr. Meier

CHRONIC PULMONARY DISEASE

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 50TH STREET

NEW YORK, N. Y. 10022

(212) 421-8985

Application for Research Grant

(Use extra pages as needed)

Date: 1/26/73

JAN 30 1973

1. Principal Investigator (give title and degrees):

Martha F. Greenwood Assistant Professor M. D.

2. Institution & address:

University of Kentucky Medical Center

Lexington, Kentucky 40506

3. Department(s) where research will be done or collaboration provided:

Department of Pediatrics

4. Short title of study:

A Study of the Functional Capacity of the Monocyte-Macrophage System.

5. Proposed starting date: July 1, 1973

6. Estimated time to complete: 3 Years

7. Brief description of specific research aims:

See appended pages

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8. Brief statement of working hypothesis:

2.

See appended pages.

9. Details of experimental design and procedures (append extra pages as necessary)

See appended pages.

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7. Brief Description of Specific Research Aims:

The aim of this project is to investigate in detail the functional capability of cells which collectively form the mononuclear phagocyte system: blood monocyte, alveolar macrophage, macrophages which have undergone in-vitro monocyte to macrophage transformation and the multinucleated giant cell of the respiratory tract.

Specifically, the objectives are:

- A) To define the phagocytic and bactericidal capacity of the normal alveolar macrophage.
- B) To compare the phagocytic and bactericidal capacity of the blood monocyte, alveolar macrophage and serially throughout the transformation process of the monocyte to macrophage in in-vitro culture.
- C) To determine if distinct receptor sites for immunoglobulins, antigen-antibody complexes and complement are present on the plasma membrane of the alveolar macrophage.
- D) To prepare an antibody whose specificity is directed toward antigenic sites on the alveolar macrophage surface and to study the effect of this anti-membrane antibody on the attachment and phagocytosis of immunologic and non-immunologic dependent particles by the alveolar macrophage.
- E) To study the effect of anti-membrane antibody on cell to cell interlinkage and differentiation of the fibroblast and alveolar macrophage into multinucleated giant cells.
- F) To quantitatively compare blood monocyte and alveolar macrophage concentrations from animals exposed to tobacco smoke inhalation and controls.
- G) To compare the in-vitro cultural characteristics and phagocytic and bactericidal capacity of alveolar macrophages lavaged from animals exposed to tobacco smoke inhalation with normal alveolar macrophage and alveolar macrophages "activated" in-vivo by an agent such as BCG.

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- H) To study the effect of smoke exposure on the plasma membrane receptor sites of the alveolar macrophage.
- I) To characterize the plasma membrane of the blood monocyte and alveolar macrophage from control and smoked animals and its alteration by anti-membrane antibody using scanning electron microscopy.
- J) To develop a method for rapid assay of bactericidal capacity and lysosomal hydrolase content of alveolar macrophages using soft agar plaque technique.

8. Brief Statement of Working Hypothesis:

The importance of the alveolar macrophage, epithelial and connective tissue barriers and the secretory activity of lining cells as pulmonary defense mechanisms is well known. The phagocytic capacity of the alveolar macrophage has been designated as the primary factor in pulmonary clearance of bacteria¹ and inhaled particulates.² Our present knowledge concerning the origin of the alveolar macrophage suggests that these cells in the normal steady state are derived from bone marrow precursors, transiently circulate in the blood as mononuclear phagocytes, emigrate through the capillary-alveolar network and subsequently undergo transformation to mature macrophages.^{3,4,5,6,7}

Although cigarette smoking has been implicated in a variety of pulmonary disorders the relationship of tobacco smoke to the monocyte-macrophage system is unclear. Previous data on the effect of cigarette smoking on the phagocytic and bactericidal capacity of alveolar macrophages is conflicting.^{8,9,10}

An assumption of this proposal is that additional data concerning the basic functional properties of the monocyte-alveolar macrophage system and its response to varied stimuli will allow a clearer understanding of the relationship between these phagocytes and enhanced or impaired pulmonary cellular defense.

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9. Details of Experimental Design and Procedures:

A) Phagocytic and bactericidal assay of normal alveolar macrophages.

Alveolar macrophages will be obtained by lavage of the pulmonary tree from male *Macaca speciosa*, stump tail monkeys, weighing 2.5-3.5 kgs. using the method of Myrvik, et. al.¹¹ Animals are sacrificed by intravenous Pentobarbital. Intratracheal pulmonary lavage is performed three times with minimal essential media (MEM) with Heparin, 25 units/ml. The cells are washed X 2 and resuspended in MEM with 10% heat-inactivated fetal calf serum or autologous serum in a final concentration of 2×10^6 /ml. Macrophages are then placed in in-vitro culture using 22 mm. glass cover slips in 35 mm. plastic tissue culture dishes or 25 ml. flat bottom tissue culture flasks. Maintenance media is MEM with 20% HIFCS and culture conditions are 37°C in a 5% CO₂-air incubator.

Phagocytic assays are performed using the following test particles: Polystyrene beads, formaldehyde treated erythrocytes, formaldehyde treated bacteria, yeast cell walls (Zymosan), fresh and effete sheep erythrocytes. The percent phagocytosis and phagocytic index will be evaluated using phase microscopy.

Bactericidal capacity will be assayed in alveolar macrophage monolayers grown in flat bottom tissue culture flasks. At the time of assay the culture media is removed and replaced with 10% pooled autologous serum, bacterial suspension and media containing 0.1% gelatin. The bacteria:macrophage ratios to be studied are 1:1 and 1:3. The flasks are incubated on a metabolic shaker at 37°C and at various time periods aliquots are removed and bacterial quantitation of the supernatant fluid is assayed by standard pour plate technique. At the same time intervals, in duplicate flasks, the macrophage monolayer is thoroughly rinsed, adherent macrophages are detached with a rubber policeman, resuspended in dilutions of distilled water for cell lysis and subsequent evaluation of intracellular bacterial survival is performed using pour plate colony

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counts. This method allows evaluation of the rate of phagocytosis of viable organisms as well as the intracellular kill capacity of the macrophage.

B) Comparison of phagocytic and bactericidal capacity of the blood monocyte, alveolar macrophage and during the monocyte to macrophage transformation in-vitro:

Peripheral blood monocytes are isolated using high M.W. Dextran sedimentation and Ficoll-Hypaque gradient centrifugation. The purified population of monocytes obtained are washed and flasks for monocyte bactericidal assay are prepared using the monocyte suspension, bacterial suspension and 20% autologous serum in MEM with 0.1% gelatin. Rotary incubation is carried out at 37°C and aliquots are withdrawn, the monocytes are lysed and bacterial quantitation is performed by standard pour plate techniques following varying intervals of incubation. The phagocytic and bactericidal capacity of the blood monocyte will be determined while in suspension, shortly after attachment to a glass surface and subsequently during the in-vitro transformation of the monocytes to macrophages as previously described.

C) Identification of receptor sites on the alveolar macrophage plasma membrane.

The presence of cell surface immunoglobulin receptor sites will be determined by evaluating the attachment and phagocytosis of opsonized erythrocytes and bacteria by the alveolar macrophage. Antibody coated erythrocytes are prepared using commercial rabbit anti-sheep hemolysin (1/1,500 dilution) by routine technique. This antibody binds to the erythrocyte by its FAB portion and leaves the Fc fragment exposed. Incubation of antibody coated erythrocytes will be used for detecting the presence of "Fc" receptor sites on the alveolar macrophage by observing surface attachment (rosette formation) and subsequent phagocytosis at 37°C. Phagocytosis of opsonized bacteria will be compared with virulent non-opsonized bacterial controls in similar experiments for identification of the

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"Fc" receptor site. The presence of a complement receptor on the alveolar surface will be sought using EAC 1,4,2,3 cells. EAC 1,4,2,3 will be prepared in an adjacent laboratory by Dr. Nancy Holland. EAC 1,4,2,3 will be incubated with monolayers of alveolar macrophages and adherence and rates of phagocytosis determined.

D) Preparation and study of the effect of an anti-membrane antibody on the alveolar macrophage.

Rabbit anti-monkey macrophage antisera will be prepared by injecting rabbits subcutaneously biweekly with a suspension of monkey alveolar macrophages combined with Freund's complete adjuvant. The macrophages are obtained by lavage and cultured in-vitro for 3-4 days. The cover slips are rinsed to remove non-adherent cells and then scraped with a rubber policeman to obtain macrophages which are then combined with Freund's adjuvant. The development in the rabbit of anti-macrophage antibodies is determined by macrophage cytolysis quantitation using fresh quinea pig complement. Since we have previously shown that the macrophage and erythrocyte surface membranes share certain antigenic components,¹² rabbit anti-monkey erythrocyte antisera will also be prepared. Rabbits are injected intraperitoneally with pure populations of washed erythrocytes weekly for 5-6 weeks and the rabbits are bled one week after injection. The anti-macrophage and anti-erythrocyte antisera are heat-inactivated, ammonium sulfate precipitated and purified using Sephadex G-200 columns. The effect of anti-membrane IgG on the primate alveolar macrophage plasma membrane can then be studied.

Phagocytosis by alveolar macrophages following 6-12 hour exposure to non-toxic concentrations of anti-membrane IgG (anti-macrophage and anti-erythrocyte) will be evaluated. Uptake of latex particles, Zymosan, formalinized bacteria and erythrocytes, antibody coated bacteria and erythrocytes and complement coated

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erythrocytes will be compared in antibody exposed alveolar macrophages and controls. These results should allow a further characterization of the alveolar macrophage plasma membrane functional capacity.

E) Long term effect of anti-membrane antibody on cells obtained by pulmonary lavage.

We have previously noted that mouse peritoneal macrophages exposed to anti-membrane antibody exhibit increased cell to cell interlinkage in long term culture. This project proposes to study in detail the effect of anti-macrophage antibody on alveolar macrophages and pulmonary derived fibroblasts. The origin and functional capacity of the multinucleated giant cell is unknown, but fusion of macrophages has been suggested. If anti-membrane antibody alters the alveolar macrophage surface and induces macrophage aggregation, membrane fusion and differentiation into multinucleated giant cells the origin of these cells would be clear and their functional capacity could then be studied.

F,G,H) Study of the functional capacity of tobacco smoke exposed alveolar macrophages.

Using the methods previously described, the in-vitro cultural characteristics, phagocytic function, bactericidal capacity and plasma membrane receptor sites will be evaluated in alveolar macrophages lavaged from animals exposed to tobacco smoke. Smoking procedures will be carried out with the cooperation and consultation of members of the Council for Tobacco Research.

I) Scanning electron microscopy studies.

This project will study the surface membrane of the blood monocyte, normal alveolar macrophage, and alveolar macrophage exposed to smoke and anti-membrane antibody. Procedures for cell preparation are similar to those previously described in SEM studies of the mammalian respiratory tract.¹³

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J) Development of soft agar plaque technique for evaluation of blood monocyte and alveolar macrophage bactericidal capacity.

Agar-gel medium has been previously used for identification of immunoglobulin secreting cells,¹⁴ for the study of blood and bone marrow colony forming units¹⁵ and for quantitative assays of serum and urine lysozyme using *Micrococcus lyso-deikticus*.¹⁶ This project proposes to attempt the development of a similar procedure for evaluation of the monocyte and alveolar macrophages' ability to break down the cell wall of various bacteria.

Blood monocytes or alveolar macrophages are added to warmed agar containing a suspension of bacteria. Aliquots are pipetted into 35 mm. plastic petri dishes and the agar media is allowed to gel at room temperature. The plates are then incubated in a 7.5% CO₂-air mixture in a humidified incubator. Zones of bacterial lysis will be enumerated using a dissecting microscope. If the technique is successful it would allow evaluation following long exposure of phagocytes to bacteria rather than rapid assay as presently required for bactericidal assays. In addition, it would allow multiple bacterial strains to be assayed against a single phagocyte population. Blood monocytes, normal alveolar macrophages, smoke exposed alveolar macrophages and BCG stimulated alveolar macrophages could be compared.

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7. Brunstetter, M.A., Hardie, J.A., Shift, R., Leurs, J.P. and Cross, C.E. The origin of pulmonary alveolar macrophages. *Arch. Intern. Med.* 127:1064, 1971.
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11. Myrvik, Q.N., Leake, E.S., and Fariss, B. Studies on pulmonary alveolar macrophages from the normal rabbit: A technique to procure them in a high state of purity. *J. Immunol.* 86:128, 1968.
12. Holland, P., Holland, N.H., and Cohn, Z.A. The selective inhibition of macrophage phagocytic receptors by anti-membrane antibodies. *J. Exp. Med.* 135:458, 1972.
13. Greenwood, M. and Holland, P. The mammalian respiratory tract surface. *Lab. Invest.* 27:296, 1972.
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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Space and research facilities for this project are available in the Pediatric Hematology research laboratory (MN 469) at the University of Kentucky Medical Center. Facilities and permanent equipment are available in this laboratory for tissue culture, microbial culture, transmission and scanning electron microscopy tissue preparation, phase, fluorescence, cinemicrophotography and routine chemical procedures. Cold room and column preparation space is available in an adjacent area (MN 441). Small animal facilities are also available in an adjacent area (MN 425) and facilities for all animals are available in the general animal quarters. A scanning electron microscope is available for use in the Department of Entomology.

11. Additional facilities required:

None.

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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CURRICULUM VITAE

Martha F. Greenwood, M.D.

B.S. Degree: University of Kentucky, 1964

M.D. Degree: University of Kentucky College of Medicine, 1968

Internship: University of Kentucky College of Medicine, 1968-69

Pediatric Residency: University of Kentucky College of Medicine, 1969-71

Fellowship: University of Kentucky College of Medicine, 1971-June 30, 1973

Assistant Professor: University of Kentucky College of Medicine, July 1, 1973

Societies and Honors:

Sophomore Book Award, 1966

R

Merck Manual Award, 1968

Outstanding Pediatric Resident Award, 1970-1971

Bibliography:

Greenwood, M.F. and Holland, P.: Scanning electron microscopy of the mammalian respiratory tract. Presented, Society for Pediatric Research, Washington, D. C., April, 1972.

Greenwood, M.F. and Holland, P.: Tracheal obstruction secondary to Histoplasma mediastinal granuloma. Chest. 62:642, 1972.

Greenwood, M.F. and Holland, P.: The mammalian respiratory tract surface. Lab. Invest. 27:296, 1972.

Greenwood, M.F. and Holland, P.: Scanning electron microscopy of the normal and BCG stimulated primate respiratory tract. J. Reticulo. Society. (In Press, publication, March, 1973).

Greenwood, M.F. and Holland, P.: Scanning electron microscopy of the normal and BCG stimulated primate respiratory tract. (Presented, Southern Society For Pediatric Research, New Orleans, January, 1973).

Greenwood, M.F., Jawad, J., Jones, E., and Holland, P.: Comparison of the bactericidal capacity of human blood phagocytes. (Submitted for presentation, Society For Pediatric Research, 1973).

Holland, P. and Greenwood, M.F.: Mitochondrial iron in sideroblastic anemias. (In Manuscript).

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CURRICULUM VITAE

Phillip Holland, M.D.

M.D.: University of Louisville School of Medicine, Louisville, Kentucky, 1954

Internship: Cook County Hospital, Chicago, Illinois, 1954-1955.

Service: R

Pediatric Residency: The Children's Hospital, Cincinnati, Ohio, 1958-1960

Fellowship: The Children's Hospital Research Foundation, Cincinnati, Ohio, 1961-1963

Instructor: University of Cincinnati, College of Medicine, 1962

Senior Research Associate: The Children's Hospital Research Foundation, Cincinnati, Ohio, 1963-1964

Assistant Professor: Department of Pediatrics, University of Kentucky School of Medicine, Lexington, Kentucky, 1964-1969

Associate Professor: Department of Pediatrics, University of Kentucky School of Medicine, Lexington, Kentucky, July 1, 1969-Present

Visiting Associate Professor: Department of Cellular Immunology, Rockefeller University, New York, New York, July 1, 1970-July 1, 1971

Scientific Societies:

REDACTED

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Holland, P. and Mauer, A.M.: Myeloid leukemoid reactions in children. *Am. J. Dis. Child.* 105:568, 1963.

Holland, P. and Mauer, A.M.: Granulocyte response in acute leukemia. Presented at meeting of Midwest Society for Pediatric Research, 1961. Abstract, *J. Ped.* 61:285, 1962.

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Holland, P., Hug, G., Schubert, W.K.: An unusual chronic reticulo-endothelial cell storage disease of childhood. Presented at meeting of Southern Society for Pediatric Research, 1964. Abstract, *Southern Med. J.* 57:1467, 1964.

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Holland, P., Jenevein, E.P., Holland, N.H.: Mechanism of lymphocyte stimulation by phytohemagglutinin. Society for Pediatric Research, 1965. (Abstract, Program, p. 94).

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Holland, P. and Scheib, R.: Rates of DNA and protein synthesis by lymphocytes from normal and agammaglobulinemic subjects. Presented at meeting of Southern Society for Pediatric Research, 1966. Abstract, *Southern Med. J.* 59:1471, 1966.

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Holland, P.: Nutrition needs and feeding problems of the handicapped child. Proceedings of the Ninth Institute on Growth and Development, 1966.

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Holland, P.: Association of increased granulocyte precursor DNA synthesis and trisomy 21. Society for Pediatric Research, 1967. (Abstract, Program, p. 81)

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Holland, P., Bobo, M.F., Shackelford, E.: Meningococchemia and intravascular clotting: The use of heparin in management. J. Ky. Med. Assoc. 66:807, 1968.

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Paglia, D.E., Holland, P., Baughan, M.A. and Valentine, W.N.: The occurrence of defective hexosephosphate isomerization in human erythrocytes and leukocytes. New Eng. J. Med. 280:66, 1969.

Abell, C. and Holland, P.: Acute toxoplasmosis complicating leukemia. Am. J. Dis. Child. 118:782, 1969.

Holland, P. and Sleamaker, K.: Motile phagocytic defense against protozoa and fungi. Presented at 6th National Meeting of the Reticuloendothelial Society, December 4, 1969.

Block, M.F. and Holland, P.: Polymorphonuclear and mononuclear leucocyte interaction with Histoplasma Capsulatum. Presented at Southern Society for Pediatric Research, November 21, 1969.

Holland, P. and Hellebusch, A.: Therapy in Wilms' tumor of childhood. J. Ky. Med. Assoc. 68:645, 1970.

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Greenwood, M.F. and Holland, P.: The mammalian respiratory tract surface: A scanning electron microscopic study. Lab Invest. 27:296, 1972. ✓

Holland, P.: Antibodies and Lysosomes, Invited lecture. Gordon Research Conference. Lysosomes, 1972.

Greenwood, M.F. and Holland, P.: Scanning electron microscopy of the normal and BCG stimulated primate respiratory tract. J. Reticulo. Society. (In Press).

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Holland, P. and Holland N.H.: Mycotic diseases: Systemic and dermatophytoses. Pediatric Therapy. Fifth edition. H.R. Shirkey, editor, C.V. Mosby, publisher.

Greenwood, M.F. and Holland, P.: Comparison of the bactericidal capacity of human blood phagocytes. (Submitted for presentation, Society For Pediatric Research, 1973).

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14. First year budget:

A. Salaries (give names or state "to be recruited")

% time

Amount

Professional (give % time of investigator(s)

even if no salary requested)

1. Martha F. Greenwood, M.D.-Principle Investigator 30%

R

2. Phillip Holland, M.D.-Co-investigator

5%

Technical

3. Senior Laboratory Technician-to be recruited

100%

7,000.00

Related Fringe Benefits (retirement, life insurance, and Social Security)

1,300.00

Sub-Total for A

\$14,900.00

B. Consumable supplies (by major categories)

Glassware

500.00

SEM Supplies

300.00

Tissue culture supplies

1,500.00

SEM Rental Time

700.00

Chemicals

500.00

Sub-Total for B

3,500.00

C. Other expenses (itemize)

Animals-Purchase and Care

1,500.00

Travel

500.00

Printing and Photography

500.00

Sub-Total for C

2,500.00

Running Total of A + B + C

20,900.00

D. Permanent equipment (itemize)

None

Sub-Total for D

0

E

3,135.00

Total request

24,035.00

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	15,800	3,500	2,500	0	3,270	25,070
Year 3	16,700	3,500	2,500	0	3,405	26,105

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Effect of Tobacco Product Inhalation on the Alveolar Macrophage. (Phillip Holland and Nancy H. Holland)	KTRB 026	44,412	7/1/72-7/1/73

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Continuation of Above	KTRB 026	Same	7/1/73-7/1/74

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

University of Kentucky Research Foundation

Mailing address for checks

MN 145, Univ. of Ky. Medical Center
800 Rose Street, Lexington, Kentucky 40506

Principal investigator

Typed Name Martha F. Greenwood

Signature *Martha F. Greenwood* Date 7/21/73

Telephone 606 233-5694
Area Code Number Extension

Responsible officer of institution

Typed Name Carl B. Delabar

Title Associate Director, U.K.R.F.

Signature *Carl B. Delabar* Date 1-25-73

Telephone 606 233-5566
Area Code Number Extension

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7/11A LAURERYS

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

September 28, 1972

Grant Application No. 741A

To: The committee comprising Drs. Loosli, Meier, and Sommers

Subject: Joseph M. Lauweryns, M.D., Ph.D., University of Leuven,
Belgium
Continuation application No. 741A
"The Lymphatics of the Lung. Their Role in Fluid Transport
and Clearance of Airborne Particulate Matter in Normal and
Experimental Conditions and in Various Lung Diseases"

History

Dr. Lauweryns has been supported, 1970 through 1972, by Grant 741 and two renewals.

Extension of the study now planned is reflected in addition of the following phrase to the title of earlier grants "... in Normal and Experimental Conditions and in Various Lung Diseases".

Application No. 741A requests \$38,806, plus two additional years. As there is no commitment, it competes as a new application.

Document Submitted (attached)

Application dated July 26, 1972 with five addenda, including Progress Report July 1971 - July 1972 and Final Report January 1970 - July 1972.

(Reprints of the major papers are on hand here, and will be sent to you if you so request).

Comment

CTR grantee Rosan was visiting professor with Lauweryns in 1970. Numerous joint publications are listed.

F.W.N.
F.W.N.

FWN:wg
Encl.

1003538569

PHYSIOLOGY OF RESPIRATORY SYSTEM and
CHRONIC PULMONARY DISEASES

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

COMMITTEE:
Dr. Loosli
Dr. Meier
Dr. Sommers

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

Application For Research Grant

AUG 14 1972

Date: July 26, 1972.

NO. 741A
NO. 741
Act: 1/1/70
Ren: 1/1/71
Ren: 1/1/72

1. Name of Investigator(s): (include Title and Degrees)

- Lauweryns, Joseph-M., M.D., Ph.D., Professor ordinarius in Microscopic Anatomy and Pathology ; Chairman, Principal Investigator.

2. Institution & Address:

- Boussauw, Luc, Lic. Biol. Sc., full-time research assistant, doctorandus. Co-investigator.

- Desmecht, Monique (Mrs. Gombeer), Lic. Biol. Sc., full-time research assistant. Co-investigator.

Experimental Laboratory of Cardiopulmonary and Genital Pathology, Department of Pathology, University of Leuven, 12, Minderbroedersstraat, B - 3000 LEUVEN-BELGIUM.

3. Short Title of Project:

The lymphatics of the lung. Their role in fluid transport and clearance of airborne particulate matter in normal and experimental conditions and in various lung diseases.

4. Proposed Starting Date:

January 1, 1973.

5. Anticipated Duration of this Specific Study:

Three (3) years.

6. Brief Description of Objectives or Specific Aims:

Despite our experience in the proposed field of investigation, numerous aspects of the structure and function of the pulmonary lymphatics are still either largely unknown or a matter of considerable controversy in the literature. Each technique of study having its shortcomings, these can only be solved accurately by a further and multidisciplinary investigation of the pulmonary lymphatics which will include various techniques, i.e. - anatomical injection studies, - stereomicroscopical studies, - serial reconstructions, - radiography and microradiography, - histological techniques, - morphometrical techniques, - histochemical techniques, - transmission electron microscopy, - freeze-etching electron microscopy and scanning electron microscopy.

During the three years of this research proposal we intend to study the pulmonary lymphatics - with the techniques proposed - along three major lines of investigation: - (1) normal morphology of the pulmonary lymphatics, - (2) an experimental study of the various morphological factors involved in the clearance and lymphatic drainage of the lung parenchyma, - (3) and morphological studies of the lung lymphatics in various pathological conditions especially in such cases where the formation of lung edema is observed (e.g. neonatal lungs: hyaline membrane disease; adult lungs: pulmonary edema, especially when associated with chronic respiratory insufficiency as in chronic bronchitis or emphysema, shock lungs (cardiogenic and neurogenic), uremia, drowning...). Though the lines of investigation are distinct, the study object (i.e. the lymphatics) is identical and the results interrelated.

These studies will necessarily end in important and original contributions which will have many basic and applied results in the structure and function of the normal and diseased lung. It is obvious that these studies are of immediate and relevant importance in biological tobacco research.

7. Give a Brief Statement of your Working Hypothesis:

A combined and multidisciplinary morphological investigation by a team of investigators working closely together since several years, will allow to avoid the shortcomings of each individual technique of investigation in lymphatic research. Studies of pulmonary fluid transport and of airborne particulate matter in normal and diseased lungs and in experimental conditions urgently request a precise and up-to-date knowledge of the pulmonary lymphatics, which is still lacking.

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8. Details of Experimental Design and Procedures: (Attach Separate Pages)

See separate pages - Addendum 1.

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

- All physical facilities are available, except item 10. Additional requirements.
- Separate list of these physical facilities - see addendum 2.

10. Additional Requirements: One scanning electron microscope.

See separate page : Addendum 3.

Biographical sketches of all principal and professional personnel (append)

See separate pages - Addendum 4.

12. List of publications: (Five most recent as pertinent) (append) and Progress Report (January 1970 - July 1972).

See separate pages - Addendum 5.

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13. Budget (1st year) (In U.S. Dollars)

A. Salaries (Personnel by names)

Professional

% time

Amount

Technical

Two laboratory technicians

100%

10,830

Sub-Total

10,830

B. Consumable Supplies (list by categories)

animals

2,260

supplies

8,000

Sub-Total

10,260

C. Other Expenses (itemize)

none

Sub-Total

none

D. Permanent Equipment (itemize)

First part-payment (= one sixth of its cost price) of a scanning electron microscope Philips, to be paid in three years - see item 10. Additional requirements - Addendum 3

17,716

none

E. Overhead (15% of A+B+C)

Total

38,806

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	11,080	10,510		17,716	none	39,306
Year 3	11,330	10,760		17,716	none	39,806

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature

Director of Project

Belgium (016) 28981

Telephone

Signature

Business Officer of the Institution

P. DE SOMER

Rector of the University

Telephone

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Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current

Title of Project	Source	Amount	Duration
Morphological studies of the Lung.	University of Leuven J.M. Lauweryns, professor, salary L. Boussauw, assistant, salary M. Desmecht, assistant, salary Salary of two technicians	\$ 12,000 \$ 6,500 \$ 6,500 \$ 11,000	yearly " " "
	Supplies and animals	\$ 5,000	"
Same area of interest since 9 years	Equipment - as listed under "physical facilities available" - from grants and local university.	This equipment will be available.	

Pending

Same project as now submitted to CTR

University of Leuven

Matching grant of the University of Leuven, defraying 50% of the purchase of the requested Scanning Electron Microscope, if CTR approves our current research proposal i.e.

1973 : first part (one sixth)
1974 : second part (one sixth)
1975 : third part (one sixth)

\$ 17,716
\$ 17,716
\$ 17,716

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ADDENDUM 1

8. DETAILS OF EXPERIMENTAL DESIGN AND PROCEDURES

1003538574

THE LYMPHATICS OF THE LUNG. THEIR ROLE IN FLUID TRANSPORT
AND CLEARANCE OF AIRBORNE PARTICULATE MATTER IN NORMAL
AND EXPERIMENTAL CONDITIONS AND IN VARIOUS LUNG DISEASES

DETAILS OF EXPERIMENTAL DESIGN AND PROCEDURES. RESEARCH PLAN

A. INTRODUCTION AND SPECIFIC AIMS

(1) Literature

1.1. As regards the normal morphology of the pulmonary lymphatic system a considerable interest has developed in recent years and much time and efforts have been spent in the study of its structure and function. Especially in the morphological field and following injection studies (Lauweryns et al., publication nr. (26), 1962, (38), 1965, (69), 1970, (89), 1971, Pennell, 1966, Pump, 1970, Trapnell, 1963, Bastianini, 1967, a,b), histological (Lauweryns et al., publication nr. (31), 1963, (34), 1964, (36), 1964, (37), 1965, (46), 1966, (60), 1968, (85), 1970, (86), 1970, (121), 1972, Aminova, 1963, 1967, Grau, 1965, Jdanov, 1969, Karpe, 1965, Kriz, 1970, Oehmke, 1968), histochemical (Borst et al., 1969, Fruschelli, 1967, 1966), radiological (Pennell, 1966, Trapnell, 1963, Lauweryns et al., publication nr. (26), 1962, (34), 1964, (69), 1970, (96), 1971) and especially electron microscopic (Borst et al., 1969, Lauweryns et al., publication nr. (57), 1968, (88), 1970, (96), 1971, (115), 1971, (124), 1972, (125), 1972, Casley-Smith, 1961, 1967, Cliff, 1970, Collan et al., 1970, Fruschelli, 1970, Kato, 1966 a, 1966 b, Klika, 1968, Kriz, 1970, Kühnel, 1966, Leak, 1966, 1967, 1968, 1968, 1970, Oehmke, 1968, Schipp, 1967, 1968, Takada, 1971, Vajda, 1971, Viragh, 1966) investigations of the lymphatic vessels of several body regions, important discoveries have been made, which have led in turn to new ideas and hypothesis concerning the functioning of the lymphatic system. In this way the existence of open junctions (Leak, 1965, 1966, 1968, 1968, 1971, Casley-Smith, 1961, 1967, 1969) and of anchoring filaments (Leak, 1966, 1967, 1968) led to the flap valve concept of the endothelial junctions (Collan, 1970). The absence of a continuous basement membrane and the presence of open junctions and cytoplasmic vesicles (Casley-Smith, 1969) explained the high permeability of the lymphatic endothelium. The presence of filaments within the lymphatic endothelial cells suggests that these cells might have an active and contractile role (Majno, 1969, Schipp, 1968). The recovery of important amounts of tracer proteins (Leak, 1970, 1971, Földi, 1955) in the lymphatic endothelium suggests that these cells might have phagocytotic properties. The ultrastructural aspect and composition of lymphatic valves also raised important ideas about their functioning (Lauweryns et al., (85), 1970, (104), 1970, (96), 1971, (115), 1971, Vajda and Tomcsik, 1971).

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Our own investigations were directed almost exclusively towards the lymphatics of the lung for several reasons. First of all, we were struck by the contradiction between the idea that the pulmonary tissue was a "dry" tissue in which no lymph was formed, as a consequence of the low pressure of the pulmonary circulation (Földi, 1964) on the one hand, and the very extensive pulmonary lymphatic plexus on the other hand (Lauweryns, publication nr. (85), 1970, (115), 1971). Secondly we realized the importance of the pulmonary lymphatic system at birth, when the fluid contents of the pulmonary airways and alveoli is apparently largely and very quickly removed via the pulmonary lymphatics (Aherne and Dawkins, 1964, Boston, 1965, Humphreys et al., 1967). Failure of this removal is somehow related to the hyaline membrane disease (Lauweryns et al., publication nr. (38), 1965, (39), 1965, (56), 1968, (70), 1968, (74), 1968, (76), 1968, (80), 1969, (88), 1970) or the idiopathic respiratory distress^{syndrome} of the newborn, a disease responsible for a large percentage of neonatal deaths (Avery, 1964). Finally the importance of the pulmonary lymphatic system for the efficiency and well functioning of respiration and vice versa is gradually becoming clear (Maier, 1966).

Our efforts were successful and we were able to extend the knowledge obtained mainly from lymphatics from skin and mesenterium (Borst, 1969, Casley-Smith, 1961, 1967, Cliff, 1970, Leak and Burke, 1968, Oehmke, 1968, Ohkuma, 1970, Schipp, 1968) and to compare them to the pulmonary lymphatics ; to confirm various aspects of their structure and to contribute with several important discoveries, such as the juxta-alveolar lymphatics and the pericentriolar filamentous bundles (Lauweryns et al., publication nr. (38), 1965, (56), 1968, (57), 1968, (60), 1968, (69), 1970), (70), 1968, (74), 1968, (76), 1968, (80), 1969, (85), 1970, (86), 1970, (104), 1970, (121), 1972, (124), (125), Lauweryns, publication nr. (26), 1962, (31), 1963, (34), 1964, (36), 1964, (37), 1965, (39), 1965, (46), 1966, (88), 1970, (89), 1971, (96), 1971, (115), 1971).

1.2. As regards an experimental approach of the various morphological factors involved in the clearance and drainage of the lung parenchyma, we have summarized the available data of the literature on the hereby included scheme 1.

A simple glance at this scheme¹ reveals that :

- no morphological data are available on the precise mechanisms of lymphatic lung drainage (except for the light optical tracer studies of Casarett et al., 1964), and that

- much contradiction exists as to the results obtained, e.g. in the all or not fagocytotic properties of the type I and type II alveolar epithelial cells, even when the investigators have used the same tracer substance (see scheme 1 for thorotrast, carbon and chinese India Ink studies).

SCHEME I

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AUTHORS	MORPH. INVEST	ANIMAL	TRACER	WAY OF ADMINISTRATION	TIME INT.	P.ALV. WALL	ALV. MACR.	LEUKOC.
DRINKER e.a. 1937	-	dog	serum protein haemoglobin egg-albumin	intratracheal injection		+		
JRTICE and SIMMONDS, 1949	-	rabbit	albumin	intratracheal instillation	9 h.			
SWANN and SPAFFORD, 1951	-	dog	various ions	intratracheal instillation		+ exc. surf		
GROSS and WESTRICK, 1954	+ (LO)	rat	india ink	intratracheal injection	4 h. à 4 d.	+	+	+
LOW and SAM- PAIO, 1957	+ (EM)	rat	thorotrast	intratracheal instillation	1/2 h.		+	
GIESEKING, 1958	+ (EM)	rat	ferric-hydrox carbon gold haemoglobin india ink	intratracheal injection	10 min. to 8 w.		+	+
KARRER, 1958	+ (EM)	mouse	india ink	intranasal instillation	1 1/2 h.		+	
POLICARD e.a. 1959	+ (EM)	rat	silicium	intratracheal injection			+	
KARRER, 1960	+ (EM)	mouse	india ink	intranasal instillation	2 h. à 1 d. 9 d.		+	+
SCHULTZ e.a. 53	-	dog	I^{131} albumin- I^{131}	intrabronchial instillation	1m. à 144h 1m. à 144h	+		
CASARETT, 1964	+ (LO)	rat	Polonium-210	inhalation			+	
CASARETT and MILLEY, 1964	+ (LO)	rabbit	$^{210}\text{Po}(\text{OH})_2$ / $^{239}\text{PuO}_2$	inhalation			+	
CASARETT and MORROW, 1964	+ (LO)	rabbit	$^{210}\text{Po}(\text{OH})_2$ ^{210}Po Polonium tagged silver	intratracheal instillation	1 à 30 d. 1 à 28 d.		+	
SCHULTZ e.a. 1964	-	isolated lung of dog	albumin- I^{131}	intrabronchial instillation	1m. à 5 h. 1h. à 24 h			
LADMAN and FINLEY, 1966	+ (EM)	dog	thorotrast	incubation of "alveolar wash"	1/2 h.			
BENSCH e.a. 1967	-	dog	I^{131} -albumin I^{131} -globulin	intratracheal instillation	15 m. à 7 d.			
DOMINGUEZ e.a. 57	-	guinea pig dog	albumin polyvinylpyr- rolidone	intratracheal injection	1/2 à 48h à 8 d.	+		
NIDEN, 1967	+ (EM)	mouse	carbon	inhalation	1/2 h.			
SANDERS and ADEE, 1968	+ (LO+ EM)	rat	polonium-210	inhalation			+	

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TYPE II CELL	TYPE I CELL	EPITH. JUNCT.	INTER- STITIUM	CONN. TISSUE	END. BV.	END. BV. JUNCT.	LUM. BV.	ENDOTH. LYMPH.	END. L. JUNCT.	LUM. LYM.	LYM. NODE
							+				
							+				
							+			?	*
			+								
-	-										
epithelial cells			+	-							
+			+	-							
+			+	-							
+			+	-							+
-	-										
+											
	+			+							
							+				
epithelial cells							+				
?			+	+						+	+
epithelial cells											
+											
epithelial cells											
+			+				+			+	
+			+				+			+	
							+				
+											
							+				
							+				
+											
	+										

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SUZUKI e.a. 1968	+	(EM)	hamster	asbestos fibers	intratracheal instillation	1 à 16 d.		+	
FAULKNER and ESTERLY, 1969	+	(EM)	rabbit (adult+ neonat.)	india ink	intratracheal injection	45 à 60 m.		+	+
ER e.a. 1969	-		dog	albumin- ¹³¹ I	intratracheal instillation	10 m. à 4 h.			
SUZUKI and CHURG, 1969	+	(EM)	hamster	asbestos fibers	intratracheal instillation	1 à 16 d. 6 à 24 mo.		+	+
CORRIN, 1970	+	(EM)	rat	carbon	intratracheal instillation	3 h. à 120h 3 h. à 48 h		+	+
DERMER, 1970	+	(EM)	guinea pig	surfactant				+	
ESTERLY and FAULKNER, 1970	+	(EM)	rabbit	india ink polystyrene	intratracheal injection	45 à 60m. 45 à 60m.		+	+
SANDERS, 1970	+	(EM)	hamster rat	PuO ₂ -239	inhalation	30 m. à 7 d.		+	
SANDERS and AD, 1970	+	(LO+ EM)	rat	²³⁹ PuO ₂	inhalation	30 m. à 7 d.		+	
BENSCH and DOMINGUEZ, 1971	+	(EM)	guinea pig	H.R.P.	intratracheal instillation	15 m. à 4 h.		+	
SANDERS e.a. /1	+	(EM)	hamster	NiO Cr ₂ O ₃	inhalation inhalation	3 d. 3 d.		+	+
SCHNEEBERGER and KARNOVSKY 1971	+	(EM)	neonatal mouse	H.R.P.	intranasal instillation	20 à 60m.			
GONZALEZ- CRUSSI and BOSTON, 1972	+	(EM)	fetal rabbit at term.	H.R.P.	intratracheal instillation	30 m. à 120 m.			

LEGEND

Morph. Invest. : Morphological Investigation

Time Int. : Time Interval (h. : hours; m. : minutes; mo. : months;
d. : days).

P. Alv. Wall : Permeability of the Alveolar Wall

Alv. Macr. : Alveolar Macrophage

Leukoc. : Leukocyte

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-	-		-				-				
(++++			+	
+	+			+							
+	+			+							
-	-			-							
	+	-	+		+	+	+			?*	
-	-										
-	+										
(+										
-	+		+		+		+				
-	+										
(+										
-	+	-	+				+				
-	+										
-	+	-	+		+	+	+				

LEGEND

Type II Cell : Type II alveolar epithelial Cell

Type I Cell : Type I alveolar epithelial Cell

Epith. Junct. : Epithelial Junction

Interstitium : Basement Membrane and Interstitium

Conn. Tissue : Connective Tissue Cell

End. BV. : Endothelial Cells of Blood Vessels

End. BV. Junct. : Endothelial Junctions of Blood Vessels

Lum. BV. : Lumen of Blood Vessels

Endoth. Lymph. : Endothelial Cells of Lymphatics

End. L. Junct. : Endothelial Junctions of Lymphatics

Lum. Lym. : Lumen of Lymphatics

Lym. Node : Lymph Node

?* : not demonstrated ; hypothetically formulated

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About one year ago (see progress report -) we have started an experimental study in this respect (using ferritin as a tracer), and it is obvious (Lauweryns and collaborators, unpublished data) that a careful experimental investigation using various morphological techniques and considering the newest literature data on lung structure and function, will yield new and basic results.

1.3. As regards morphological studies of the lung lymphatics in various pathological conditions of the lung parenchyma, it may be stated that no real data are available in the literature, except for casual annotations and our earlier histological (Lauweryns et al., publication nr. (38), 1965, (39), 1965) and morphometrical (Lauweryns et al., publication nr. (56), 1968, (80), 1969) studies on the lung lymphatics in neonatal hyaline membrane disease (76), 1968, (88), 1970) and in drowning (Lauweryns, (92), 1970).

Still the formation of lung edema occurs daily in medical practice in a large number of patients, in association e.g. with chronic and progressive respiratory insufficiency as in chronic bronchitis or emphysema, shock lungs (cardiogenic and neurogenic), uremia, drowning...

It does not seem believable that (almost) nothing is known about the fine morphology of the lung lymphatics in diseased and edematous lungs.

(2) Aims and rationale

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From the foregoing we feel that our studies on the lymphatics of the lung should be carried on, as they are not only basically important but are also directed to understand the diseased lung better.

The aims of the proposed study are indeed :

2.1. A further and thorough morphological investigation of the lymphatic system of normal lungs, i.e.

- High power electron microscopy and enzyme digestion studies of anchoring and endothelial filaments.
- Comparative electron microscopic investigations of capillaries, collecting ducts and conducting channels in various animal species and body localizations.
- Electron microscopic and scanning electron microscopic studies of the "roots" of the pulmonary lymphatic system and the valves.
- The innervation of the lung lymphatics.

2.2. An experimental approach of the various morphological factors (especially the lymphatics) involved in the clearance and drainage of the lung parenchyma of airborne particulate matter.

- 2.3. A morphological study of the lung lymphatics in lung edema and in various diseases (chronic progressive respiratory insufficiency as in chronic bronchitis or emphysema, shock lungs (cardiogenic and neurogenic), uremia, drowning.

These three areas 2.1., 2.2. and 2.3. will be investigated in parallel during the three years of our research proposal.

2.1. Morphology of the pulmonary lymphatic system

The most urgent questions to be solved in this field are :

2.1.a. The nature and the function of the anchoring filaments.

As it is up to now virtually impossible to isolate these filaments and to submit them to chemical analysis, their nature and function might perhaps be discovered by a high resolution EM in order to compare them with other well known filaments like actin, myosin, protocollagen, combined with enzyme (hyaluronidase, elastase,...) digestion studies. One may also wonder about the nature and the function of the filaments situated within the lymphatic endothelial cells (Cecio, 1967). In a similar way high power electron microscopy for comparative reasons combined with enzyme digestion methods and the study of their morphological reaction towards pharmacological agents might reveal their nature and function.

2.1.b. The nature and the function of the very recently discovered pericentriolar filamentous bundles (Lauweryns et al., (124), (125), in press). A study of their distribution in different cell types and in various animal species might at least reveal whether they are specific to some animals, to some cell types and might hence suggest possible roles. If they were specific to lymphatic endothelium and present in various species, their role would certainly be related to lymphatic function.

2.1.c. The endings of the pulmonary lymphatic system. Are the terminal divisions of the small lymphatic capillaries (the initial lymphatics, Casley-Smith, 1961) blind fingerlike projections, on themselves closed loops, or is there fine communication with tissue clefts (Klika, 1968) ? This problem can best be studied by the use of electron microscopic techniques which allow a certain degree of tridimensional visualization and surface view, i.e. freeze-etching and especially scanning electron microscopy.

2.1.d. The pulmonary lymphatic valves. Our graphic reconstructions (Lauweryns et al., publication nr. (85), 1970, (86), 1970, (89), 1971, (96), 1971, (121), 1972) and stereomicroscopic observations (Lauweryns, publication nr. (111), 1971, (115), 1971) of pulmonary lymphatic valves could most adequately be confirmed and extended by scanning electron microscopy of these valves.

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2.1.e. The morphological differences between lymphatic capillaries, collecting channels and conducting channels. It is hardly believable that no data are known concerning this subject. The answer to this question implies a thorough electron microscopical and histological observation of a large number of lymphatics in various localizations and in various animals.

2.1.f. The innervation of the lung lymphatics and their valves. (Gellert, 1967, Kubik, 1955, Melnikova, 1964, Schipp, 1965, Shdanow, 1967, Vajda, 1966). Here a combined neurohistochemical and electron microscopic investigation - as we have executed in other areas (Lauweryns et al., publication nr. (84), 1969, (106), 1970, (108), 1971, (117), 1972) - will lead to an answer.

Rationale : These various morphological problems are to some extent related and can also only be accurately solved (Lauweryns et al., publication nr. (86), 1969, (96), 1971) by a combined and multidisciplinary approach including various techniques, which are familiar to us, i.e. : - anatomical injection studies, - serial reconstructions, - radiography and microradiography, - histological techniques, - morphometrical studies, - histochemical techniques and - electron microscopy.

All these techniques currently used at our laboratory have indeed their limitations, and some of them could even produce artefacts. Injection of the lymphatics of the pleura i.e. with radioopaque substances followed by radiography, or with plastic substances followed by corrosion of the tissues, easily causes disruption of the delicate walls of the smaller lymphatic vessels and filling of tissue clefts. The injection moreover may inverse valves and hence result in false pictures of lymphatic drainage-pathways. Corrosion casts and radiographs moreover do not reveal the relationship of the vessels to the surrounding tissues in fine detail.

Histological examination i.e. of non injected lymphatics is hindered by difficulties in recognizing pulmonary lymphatic capillaries and in differentiating them with certitude from smallblood vessels and tissue clefts. Histochemical methods to differentiate lymphatic vessels from other structures are not known.

Reconstructions from serial histological sections (Staubesand et al., 1953, Comparini et al., 1965, Boussauw et al., publication nr. (85), 1970) are subject to the same difficulties and have moreover the disadvantage of being a time consuming procedure.

Electronmicroscopy is also limited because only very small areas can be investigated.

Freeze-etching electron microscopy will also be applied. This technique is different from traditional electron microscopy because it avoids some chemical interactions in the tissues, exposes tissue structures in relief and allows the observation of membrane surfaces "en face". We are thoroughly acquainted with

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this technique, having already done extensive studies on the lung parenchyma (Lauweryns et al., publication nr. (93), 1971, (97), 1970, (100), 1971, (128), submitted). In studying the lymphatics, it still poses one major problem as basal membranes are not visible on freeze-etch electron micrographs (hence hindering the differentiation from lymphatic capillaries with other small vessels or even tissue clefts).

Scanning electron microscopy will also be executed. It will certainly add important information and new, original and unique data to our field of study; indeed it allows the investigation of relatively large samples (as compared to transmission electron microscopy) both at low and higher magnification and with great focal depth. Moreover the peculiar tridimensional "geometrical" structure of the lung seems to be ideally adapted for SEM investigation.

From the limitations and inherent risks of each of these methods, we feel that only a multidisciplinary investigation by applying various methods of investigation can result in a clear, synthetic and true picture of the pulmonary lymphatic system, as already stands out from our earlier work (Lauweryns et al., publication nr. (26), 1962, (31), 1963, (34), 1964, (36), 1964, (37), 1965, (38), 1965, (39), 1965, (46), 1966, (56), 1968, (57), 1968, (60), 1968, (69), 1970, (70), 1968, (74), 1968, (76), 1968, (80), 1969, (84), 1969, (85), 1969, (86), 1970, (88), 1970, (89), 1971, (96), 1971, (104), 1970, (106), 1970, (108), 1971, (111), 1971, (115), 1971, (117), 1972, (121), 1972, (124) & (125) in press).

2.2. Experimental study of the various morphological factors (especially the lymphatics) involved in fluid transport and clearance of particulate matter

Following the pathway(s) which free particles have to follow to reach the lymphatic capillary lumen from the alveolar lumen, the following factors have to be considered: - alveolar macrophages, - alveolar epithelium, - basal membrane(s), - alveolar interstitium with blood capillaries, - lymphatic endothelium.

2.2.a. The alveolar macrophages, whose origin is seriously controverted (Bertalauffy and Leblond, 1953, Karrer, 1960, Bowden et al., 1968, Pinketi et al., 1966) do represent an important factor in alveolar clearance (Karrer, 1958, Policard et al., 1959, Faulkner and Esterly, 1969, Sanders and Adey, 1970). They are sometimes aided in their task by leucocytes which may be present in the alveolar lumen as free cells (Gross and Westrick, 1954, Giesecking, 1958, Karrer, 1960, Suzuki and Churg, 1969, Faulkner and Esterly, 1969). After resorption of foreign material the macrophages may (Cole, 1944, Vorwald, 1950, Karrer, 1960, Davies, 1963) or may not (Gross and Hatch, 1962, Hatch and Gross, 1964, Casarett and Milley, 1964) migrate to the interstitium and further on to the lymphatics.

These different aspects should be investigated.

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2.2.b. The phagocytotic properties of the alveolar epithelium. - As clearly demonstrated in table 1, extreme contradictions exist in the literature concerning the phagocytotic properties of the alveolar epithelium ; this problem is not only important as such, but also because it remains related to the cellular origin of the surfactant (Macklin, 1954, Kikkawa et al., 1965, Niden, 1967, Kuhn, 1968, Azzopardi and Thurlbeck, 1969, Dermer, 1970 a).

2.2.c. The permeability of the basal membrane(s) which are not observed on freeze-etching electron micrographs (Lauweryns et al, publication nr. (93), 1971, (97), 1971, (100), 1971, (128) submitted ; Friederici, 1968), also remains an open question (Schneeberger-Keeley and Karnovsky, 1968).

Precise studies with high resolution electron microscopy on the fate and migration of particles through the interstitium and their interaction with the connective tissue cells have also not been executed.

2.2.d. The way(s) by which particles enter the lymphatic lumen is mainly speculative (Heppleston, 1963). An intercellular transport via typical open junctions is usually accepted (Lauweryns et al., publication nr. (73), 1969), still a transcellular transport may occur as well (Casley-Smith, 1965 ; Leak and Burke, 1968).

It is also unsettled if the lymphatic endothelial lining cells are capable of storing, digesting or transporting particles ?

The same question may be asked concerning the blood capillaries.

Though both vessel systems (blood- and lymph vessels) probably play a role in alveolar clearance, their relative importance remains unsettled (Meyer et al., 1969, Dermer, 1970 b, Leak, 1970, 1971).

Rationale : Though using in general terms the same spectrum of techniques, as mentioned under 2.1., this investigation will be mainly electron microscopical.

2.3. The lung lymphatics in various diseases

Being thoroughly acquainted with the lymphatics in the normal adult and infant lung and having approached them already in two diseases (i.e. Hyaline Membrane Disease of the human newborn (Lauweryns et al., publication nr. (38), 1965, (39), 1965, (56), 1968, (76), 1968, (80), 1969, (88), 1970) and drowning (Lauweryns, publication nr. (92), 1970), we propose to investigate them in various diseases (chronic and progressive respiratory insufficiency as in chronic bronchitis or emphysema, shock lungs (cardiogenic and neurogenic), uremia, drowning, hyaline membrane disease, lungs of other neonatal deaths), especially when these are accompanied with edema formation.

As practically nothing is known about this subject, it will remain a delicate task and constitute quite an endeavour ; it is however obvious that we are ideally prepared to investigate this challenging problem, being thoroughly acquainted with the normal anatomy of the lymphatics and being trained as a pathologist.

Rationale : Gross (including lymphatic injections) and routine microscopic studies (including morphometric pilot studies) of the lungs will be first executed. Depending on the obtained results further investigations (transmission electron microscopical, scanning electron microscopical, freeze-etching) will be furthermore undertaken.

B. METHODS OF PROCEDURE

(1) Species to be investigated

1.1. As regards the normal morphology of the pulmonary lymphatic system :

- rabbit (newborn and adult).
- human infant and adult lungs.

We are thoroughly acquainted with rabbit and human lungs, as we are studying them since 1958 (cfr. curriculum vitae).

1.2. As regards the experimental study :

- newborn rabbits.

We are indeed not only thoroughly acquainted with their lungs, but have already undertaken these experiments since one year with satisfactory results.

1.3. As regards the lung lymphatics in various diseases :

Their will be no problem of collecting lungs as we have currently about 120 infant and 350 adult postmortems yearly.

As regards electron microscopy, we have no problem to be immediately alerted and do the fixations as soon as possible ; we are indeed immediately informed by the "Newborn premature center", the "High care unit" and the "Clinic for chronic lung diseases". The efficiency of this delicate cooperation has been demonstrated in our earlier electron microscopical studies of "Hyaline Membrane Disease" and human "Lung Lymphatics" in normal lungs (cfr. publications - curriculum vitae).

It is clear from the above that we will have no problems to harvest the lungs needed for our investigation.

(2) Techniques to be applied to :

2.1. Normal morphology of the pulmonary lymphatic system :

The techniques to be applied are all familiar to us (enzyme digestion excepted). They are especially :

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- high power electron microscopy (problems 2.1.a ; 2.1.b ; 2.1.e ; 2.1.f).
- high power electron microscopy and enzyme digestion (problems 2.1.a ; 2.1.b ; 2.1.e ; 2.1.f).
- freeze-etching electron microscopy (problems 2.1.d ; 2.1.c).
- scanning electron microscopy (problems 2.1.d ; 2.1.c).
- electron microscopy (problems 2.1.b ; 2.1.e).

As regards the innervation of the pulmonary lymphatics, ^{this} will be studied by the classical histological techniques of silver impregnation (Bodian-Van Campenhout), by histochemical studies on cholinesterases (method of Koelle, modified by Gerebtzoff (1959) and by freeze-drying-fluorescent studies on catecholamines (method of Falck, 1962, 1965). We are thoroughly acquainted with these techniques (cfr. Lauweryns et al., publication nr. (59), 1967, (64), 1969, (84), 1969, (106), 1970, (117), 1972).

For a distinction of cholinergic and adrenergic nerve endings, we will apply the methods of Richardson (1966), De Robertis and Pellegrino, Wood and Barnett, (1964). Here also high resolution electron microscopy is best applied.

2.2. Experimental study :

- This will be the continuation of our study, started about one year ago. Under slight anesthesia, a tracer (0,05 cc) will be intratracheally instilled. Instillation causes indeed much less tissue disturbance or damage than injection. The amount of tracer introduced in the lungs by this procedure is also constant ; this is not true in cases of intranasal instillation.

- The animals are killed at various intervals after the instillation (from 15 min. to 24 days) and a careful electron microscopical investigation of both lungs carried out.

- Morphometric studies will be carried out to estimate the removal of the tracer and its fine localization in the lung.

- As tracer we will first use a colloidal solution of ferritin (\emptyset 100 à 110 Å, MW + 465000, 2 X cristalline, cadmium free, N.B.C., Cleveland) ; Indeed ferritin is not only a widely used and efficient tracer (biological protein, electrondense, non-toxic, Bruns and Palade, 1968) but has also never been used in an analogous study.

- To check the probable influence of the physico-chemical properties of the tracer ferritin, we will next use a totally different tracer, i.e. a carbon suspension (C11/1431 a; Günther Wagner, Hanover) whose composition has been described by Biozzi et al., (1953).

- Both series of results will be compared.

- After these studies using ferritin and a carbon suspension, other tracers could be used, if necessary (i.e. horseradish peroxidase ; thorotrast ; colloidal gold : etc).

2.3. The lung lymphatics in various diseases :

As this constitutes an entirely new investigation with no reliable literature data available, we will apply the whole spectrum of morphological techniques which we have used earlier to study the lymphatics in normal lungs. Indeed and as explained in Rationale 2.1., each technique having its shortcomings, a combined and multidisciplinary approach is inevitable including various methods, which are familiar to us : - anatomical injection studies, - serial reconstructions, - radiography and microradiography, - histological techniques, -morphometrical techniques, - histochemical techniques, - transmission electron microscopy, - freeze-etching electron microscopy and scanning electron microscopy.

The "pilot approach" will include detailed gross and microscopic studies, combined with some injection studies and elementary morphometry.

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ADDENDUM 2

9. SEPARATE LIST OF PHYSICAL FACILITIES AVAILABLE

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ADDENDUM 2

List of physical facilities available

- a) for histology :
 - all routine equipment : drying-stoves, rotary microtome Spencer, microtome for large sections Tetrande Jung, 3 binocular microscopes, 2 stereomicroscopes.
- b) for radiography :
 - radiographical apparatus Balteau with image intensifier.
 - microradiographical apparatus Baltodyne, Balteau-General Electric.
- c) for histochemistry :
 - cryostat Pearce.
 - freeze-drying apparatus Pearce..
- d) for morphometry :
 - one microscope with lateral projection arm.
 - one Olivetti calculator.
- e) for electron microscopy :
 - one ultramicrotome Porter Blum MT-2.
 - one automatic ultramicrotome Reichert.
 - one vacuum-coating apparatus Edwards.
 - dark room facilities.
 - one freeze-etching ultramicrotome Balzers.
 - one electron microscope Zeiss Em 9 (resolution $\pm 12 \text{ \AA}$)
 - one electron microscope Philips EM 300 (resolution $\pm 3 \text{ \AA}$).

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ADDENDUM 3

10. ADDITIONAL REQUIREMENTS

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ADDENDUM 3. Item 10, ADDITIONAL REQUIREMENTS

We have no scanning electron microscope available at our laboratory, but have been thoroughly introduced to this technique and discipline and carried out some preliminary work at the Faculty of Sciences and Engineering at the University of Ghent. However, the submitted research program in Scanning Electron Microscopy far exceeds the "occasional" possibilities we have to work in Ghent, which is furthermore situated at a distance of about 100 miles from Leuven (hence hindering considerably the efficiency of our work).

Moreover we cannot carry out an ideal preparation of our tissues at Ghent as we are not allowed there to execute a "critical point drying" of the lung tissues (which is however best and necessary for Scanning Electron Microscopy), but only "air drying" which badly cracks the lung tissues, causes many artefacts and produces very often poor results. To investigate the ultrastructure of the delicate lung lymphatics and obtain reliable results, it is absolutely necessary to have a Scanning Electron Microscope continuously available at the laboratory to study the specimens without artificially induced artefacts.

Hence we have contacted the Philips Company who has offered us the "Philips PSEM - 500 Quantitative High Performance Scanning Microscope", cost price (tax included) : \$ 106,296. They have allowed us to pay the instrument in three yearly payments without rent on the due sums in the meantime.

We have then contacted our local university, which will consider to intervene for 50% in the cost price, if CTR first approves our research proposal.

Hence the payments will be as follows :

- i.e. : - first year : one third : \$ 35.432 : - CTR : \$ 17,716 (one sixth)
- University of Leuven : \$ 17,716 (one sixth)
- second year : one third : \$ 35.432 : - CTR : \$ 17,716
- University of Leuven : \$ 17,716
- third year : one third : \$ 35.432 : - CTR : \$ 17,716
- University of Leuven : \$ 17,716

The price includes the installation in our laboratory. We have given the preference to the Philips instrument because we have already a Philips transmission electron microscope and hence know the excellent local maintenance service of this company in Belgium. Moreover the main factory is in Holland, hence very close to our laboratory, if a problem should arise. The same cannot be said in our particular case for other companies, which - as far as scanning electron microscopy is concerned - only have a local sale representation but no real technical service available.

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903 MENDICINO

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 1, 1973

5
Rejected

Grant Application No. 903

To: The committee comprising Drs. Cattell, Gardner and Loosli

Subject: Joseph Mendicino, Ph.D., University of Georgia, Athens, Ga.
New application No. 903
"The Role of Nicotine, 3', 5'-Cyclic AMP and Related
Enzyme Systems in Pulmonary Function and Disease"

History

Recently the applicant telephoned at the behest of CTR grantee James Travis in the same university and department. In view of the January 31 closing date, Dr. Mendicino elected to submit a full application, rather than following the preferred route of a preliminary informal inquiry.

The request is for \$31,230, plus two additional years.

Documents Submitted

Attached is the application dated January 30, 1973 (17 pages).

(Reprints or galley proofs of recent papers cited in the application were provided; copies will be made and forwarded if you wish.)

Comment

We are obtaining an evaluation from CTR grantee Wayne L. Ryan who is investigating the same enzyme system.

F.W.N.
F.W.N.

FWN:wg
Encl.

1003538604

TO: Dr. Frederic W. Nordsiek

FROM: Dr. Wayne L. Ryan

DATE: February 5, 1973

SUBJECT: Mendicino Grant Application

#903

The hypothesis of this application is not clear and the introduction consists of a series of statements which are not tied together. For example, the last paragraph on page 8 doesn't seem to have any relation to the problem.

The documentation in the introduction, specific aims, and methods is very poor. Several hundred papers have been written on catecholamine activation of adenylate cyclase, and he doesn't reference them so as to indicate familiarity.

The first step in the experimental plan is to measure the levels of cyclic AMP in lung and brain of animals treated with nicotine.

The methods section indicates that he plans to use his method (which has not been published and is not adequately described in the proposal) for assaying cyclic AMP.

Aside from the lack of verification of his method, there is little reason for using it. Only a few milligrams of tissue are required for the radioimmunoassay. The samples should be purified before assaying and phosphodiesterase controls used to authenticate that the material measured is cyclic AMP.

The second step in the experimental plan is to measure adenylate cyclase and phosphodiesterase after administration of nicotine.

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The adenylate cyclase method he describes is not his method, but a modification of the method of Krishna and should be referenced.

The PDE method is that of Thompson and Appleman. The adenylate cyclase measurement is extremely difficult, and there is no indication that he has actually used it.

If nicotine elevates the level of catecholamines in blood, he can be assured of higher cyclic AMP levels in the tissues. An interesting question is whether nicotine can act directly on adenylate cyclase. That is, is there an adenylate receptor in cells that is responsive to nicotine? This experiment could be conducted in vitro using cell homogenates and would answer the most important question that he asks.

In general, the proposal is poorly written, but the most disturbing part of it is the use of his own methods which have not been reported. Examination of his curriculum vitae would suggest that the problems I have pointed out are due to lack of experience and knowledge of the area.

WLR/mm

bc (by TR)
DRS. Gattell
Gantner
Hoosli
Hockett
Joumays

1003538606

Comm.

Dr. Cattell
Dr. Gardner
Loosli

CHRONIC PULMONARY DISEASES

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 50TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant.
(Use extra pages as needed)

Date: Jan. 30, 1973

1. Principal Investigator (give title and degrees):

Joseph Mendicino, Ph.D., Associate Professor

Hussein Abou Issa, Ph.D., Senior Research Associate

2. Institution & address.

Department of Biochemistry

University of Georgia

Athens, Georgia 30602 Phone: 404-542-1334

3. Department(s) where research will be done or collaboration provided:

Department of Biochemistry

University of Georgia

Athens, Georgia 30602

4. Short title of study:

The Role of Nicotine, 3', 5'-Cyclic AMP and Related Enzyme Systems in
Pulmonary Function and Disease

5. Proposed starting date: June 1, 1973

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

The principal aim of the proposed study will be to investigate the characteristics of the enzyme systems in lung and brain which synthesize cyclic adenosine 3', 5'-monophosphate (cyclic 3', 5' AMP) in response to nicotine and its metabolites, and to examine the effects of nicotine and cyclic 3', 5' AMP on the multienzyme systems which are, in turn, influenced by these compounds. The specific objectives will be (1) to study the mechanism of action of nicotine on the synthesis of cyclic 3', 5' AMP and related enzyme systems in lung and brain, (2) to determine the levels of cyclic 3', 5' AMP in control and nicotine treated tissues "in vivo" and in isolated tissues and slices, (3) to determine the activities of adenylylase and cyclic 3', 5' AMP phosphodiesterase in control tissues and in tissues treated with nicotine, (4) to examine the effects of nicotine, its metabolites and analogues on hormone responsiveness of control and treated cells derived from lung and brain tissues.

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8. Brief statement of working hypothesis:

2.

See attached

9. Details of experimental design and procedures (append extra pages as necessary)

See attached

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The research will be carried out in the Department of Biochemistry at the University of Georgia. The laboratory is completely equipped with instruments necessary for the studies described in this proposal. Equipment which is available and will be used during this investigation include:

Sorvall refrigerated preparative centrifuges (5), Spinco preparative ultracentrifuges (4), Spinco Model E analytical ultracentrifuge, Zeiss PMQ spectrophotometer, walk-in cold rooms and 30 and 37 incubators adaptable to higher and lower temperatures, Beckman amino acid analyzer, Model 120C (2), paper electrophoresis facilities, Beckman pH meters, fraction collectors, Waring blenders, homogenizers, facilities for housing of experimental tissues and laboratory animals with well equipped surgical room, tissue culture facilities and equipments. Isotope counting and scanning equipment, including two Packard scintillation counters, Gilford spectrophotometer, model 2000 (2), Gilford spectrophotometer model 240, Beckman DU and DBG, Cary spectrophotometers, model 14 and 15, ultrasonic cell disruptor, lyophilizers and evaporators and the usual equipment for chromatography and other ordinary laboratory procedures. The University has also a general electron microscopy facility having both scanning and transmission microscopes. The procurement of the necessary tissues can be easily arranged. The department has facilities for keeping small laboratory animals. The principal investigator has a good working arrangement with local packing houses, so that large amounts of fresh lung tissue can be obtained from larger animals. Human lung tissue may be obtained at lobectomy from several local hospitals.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

See attached

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See attached

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14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Joseph Mendicino	30	0-
Hussein Abou-Issa	100	0-
Fringe Benefits (14%)		
Technical		
Gerald Hickman	50	
Donald McRorie	50	
Fringe Benefits (6%)		
Sub-Total for A		18,450

B. Consumable supplies (by major categories)

Chemicals, glassware	2,000	
Isotopes	1,500	
Tissues and care of experimental animals	750	
Sub-Total for B		4,250

C. Other expenses (itemize)

Travel to National Meetings	600	
Publication and reference material	900	
Sub-Total for C		1,500
Running Total of A + B + C		24,200

D. Permanent equipment (itemize)

RC2B Refrigerated Centrifuge and Heads	3,400	
Sub-Total for D		3,400

E. Indirect costs (15% of A+B+C)

E	3,630	
Total request		31,230

15. Estimated future requirements:

	Salaries	Consumable Suppl	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	13,500	4,500	1,500	2,500	2,925	24,925
Year 3	15,000	4,000	1,500	2,500	3,075	26,075

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Enzyme Interactions in Metabolic Regulation	N.I.H.	\$21,805	06/01/72-05/31/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
The Biosynthesis of Cell Wall Polysaccharides	N.S.F.	\$116,925	06/01/73 to 05/31/76

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Joseph MendicinoSignature Joseph Mendicino Date Jan 29, 1973Telephone 404 542-1334 22
Area Code Number Extension

Responsible officer of institution

Typed Name Robert C. AndersonTitle Vice President for ResearchSignature Robert C. Anderson Date _____Telephone 404 542-3360
Area Code Number Extension

Checks payable to

The University of Georgia

Mailing address for checks

Alan W. Barber

Acting Vice President for Business &
FinanceThe University of Georgia

Athens, Georgia 30602

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8. Brief Statement of Working Hypothesis - The exact mechanism by which nicotine exerts its effects on various mammalian tissues is not yet well understood. It is not certain whether it directly increases or decreases the rate of formation of intracellular cyclic 3',5'-AMP under different physiological conditions and thereby controls the rate of specific metabolic processes, or whether the possible secondary effects of nicotine in increasing the levels of norepinephrine, serotonin or dopamine in brain tissues for instance, indirectly stimulates or inhibits the synthesis or breakdown of cyclic 3',5' AMP. Two tissues will be used in preliminary studies, lung tissue, which along with kidney and liver, metabolizes nicotine, and brain tissue which does not degrade this compound. To examine the role of nicotine we propose to utilize a technique for the direct measurement of cyclic 3',5'-AMP levels in slices and various tissues preparations of lung and brain. The possible influence of nicotine on the activities of adenylcyclase and cyclic 3',5' AMP phosphodiesterase in these preparations will be examined. The cyclic 3',5' AMP dependent protein kinases of lung and brain will be isolated and their function in the regulation of specific glycolytic enzymes which change quickly in response to increased levels of nicotine will be evaluated. The working hypothesis of the proposed study is based on the idea that in order to understand the primary effects of nicotine it is necessary to study the properties, interactions and regulation of the enzyme systems which are influenced by the presence of nicotine.

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9. Details of Experimental Design and Procedures.

1. Background

The physiological disposition of C^{14} -labeled nicotine, studied by autoradiographical techniques, showed that nicotine was rapidly distributed throughout the tissues and body fluids. Present evidence indicates that nicotine and/or its metabolites are concentrated in target organs such as the brain, adrenal medulla, lungs, liver and the superior cervical ganglion (1). High concentrations of nicotine accumulate very rapidly in the brain of the guinea pigs after the administration of C^{14} -labeled nicotine (2). Also after intratracheal administration of C^{14} - nicotine, a large amount of radioactivity appears in the lungs (1).

Several studies have been done on the pharmacology of nicotine and smoking as well as the release of catecholamines through the action of nicotine. Silvette et al (3) have shown that the dosage of nicotine derived from smoking was sufficient to cause a release of epinephrine from the adrenal medulla. Westfall and Watts (4) also studied the effects of cigarette smoking on epinephrine secretion in the dog and came to the same conclusion. Several studies (5) have also shown the increase in brain epinephrine following nicotine administration. A great deal of significance was placed on the fact that inhalation of cigarette smoke produced an increase in epinephrine levels of peripheral arterial blood since this is the blood which reaches all tissues (6). Epinephrine and total catecholamine output progressively increase above control levels during periods of heavy smoking and these levels return to normal soon after smoking is stopped (7,8). Several possibilities for the mechanism of amine release by nicotine have been discussed (9). The release could be produced by inhibition of an active storage process or by enhancement of the permeability of the cell membrane making it permeable to various amines in both directions. It has been suggested in several studies (10,11) that smoking in man and nicotine administration in dogs is followed by increased plasma concentrations of free fatty acids. Isotopic studies (12) have shown that the rise is due to an augmented influx of free fatty acids into the circulation. It has been suggested that the increased sympathetic and catecholamine activity associated with the presence of nicotine have a basic role in the free fatty acid response (8,13).

The effect of smoking and nicotine on blood glucose levels was also examined. The results of some studies indicate that nicotine causes a significant increase in blood glucose levels (14,15). The hyperglycemic effect of nicotine and smoking was attributed to an increased epinephrine secretion (16). However, several other studies have shown no significant differences in the blood glucose levels of smokers and non-smokers (17). The molecular action of nicotine and the exact mechanism by which nicotine may affect these biochemical processes is still obscure and clearly needs further study at the molecular level. Several of these processes such as the increased catabolism of carbohydrate as well as the increased lipolysis and release of fatty acids are also elicited by administration of 3',5' cyclic AMP. Cyclic AMP is also thought to be involved in the release of hormones such as insulin, thyroxine, prostaglandins and anterior pituitary hormones.

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It is therefore possible that nicotine acts directly or indirectly on specific enzyme systems leading to an increased or decreased production of 3',5' cyclic AMP. Cyclic AMP, in turn, might then activate cyclic AMP-dependent protein kinases that in turn stimulate glycogenolysis and thereby raise blood sugar levels. These effectors may also activate a triglyceride lipase at the same time causing increased mobilization of free fatty acids. Increased cyclic AMP levels may also promote the release of epinephrine and histamine in the lungs. An epinephrine-sensitive adenylyl cyclase has been identified in the subcellular fractions of the lungs (17) and phosphodiesterase is also present (18). Recently Cyclic AMP has been found to be associated with bronchial asthma (19) and caffeine and epinephrine have been found to act synergistically on increasing cyclic AMP levels in rat lung slices (19). The action of nicotine might be due to a direct effect on the enzyme systems responsible for Cyclic AMP homeostasis in the cell leading to an increased or decreased production of 3',5'-Cyclic AMP, or to an indirect effect through the release of epinephrine. The principal objectives of this study are to determine the site and the molecular action of nicotine by studying the effects of nicotine on the levels of 3',5'-Cyclic AMP as well as on the activities of the enzyme systems responsible for the synthesis and degradation of Cyclic AMP in lungs and brain. An attempt will be made to correlate the relationship between the levels of 3',5'-Cyclic AMP and the activity of the enzymes associated with carbohydrate and lipid catabolism in these tissues.

The coordinated function in living systems requires that the cell maintain homeostasis under varying physiological conditions. These mechanisms must in turn depend on the ability of multienzyme systems to increase or decrease the rates of metabolic processes. An understanding of the factors which influence and regulate these enzyme systems is necessary in order to understand the ability of the tissue or organism to maintain this constantly changing dynamic equilibrium. Nicotine and hormonal factors may exert their regulatory effects by simultaneously stimulating and inactivating specific enzyme reactions involved in the breakdown or synthesis of glucose and glycogen and fat. The ability of various protein kinases and protein phosphatases to alter the activity of key rate limiting enzymes such as fructose 1,6-diphosphatase, glycogen synthetase, phosphofructokinase and phosphorylase will be evaluated. The influence of nicotine on the regulatory enzymes will be being examined to determine which enzyme-enzyme interactions are involved in the regulation of these metabolic pathways.

This system in liver and kidney is capable of responding to stimuli which evoke a response to satisfy extracellular energy requirements and cause stored glycogen or hexose derived from the breakdown of protein to be released as free glucose into the circulatory system. Likewise, the same system must satisfy numerous intracellular energy requirements by degrading glycogen or glucose to pyruvate. Factors which cause a specific biosynthetic process to occur in the cell must also simultaneously stimulate the catabolism of carbohydrate or fat by just enough to satisfy this extra energy requirement. One multienzyme system which often supplies this additional energy requirement is the glycolytic system. The basic question which we will be concerned with in the present studies is how do numerous hormones and nicotine regulate the rate of these multienzyme systems in lung and brain tissue. How is the rate of formation of high energy intermediates by a relatively few catabolic systems coordinated with the rates of the many

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synthetic systems which utilize energy in the cell.

Two 3',5' cyclic AMP-dependent and independent protein kinases have been isolated from kidney extracts in order to study the regulation of glycogen synthetase and phosphorylase in kidney and mammary gland. These two kinases were purified from the soluble and the subcellular membrane fractions of swine kidney, and mammary gland. The soluble kinase is dependent on 3',5' cyclic AMP for activity. Under the conditions employed, the K_a value for cyclic AMP for glycogen synthetase I kinase activity was in the range of 60-70 nM. The activation was quite specific for cyclic AMP.

As a working hypothesis in our present studies we assumed that hormones or other agents such as nicotine direct biological processes causing a general increase in catabolic metabolism via 3',5' cyclic AMP in order to satisfy the energy requirements of the specific synthetic processes which they stimulated. The graded response of both catabolism of fat and carbohydrate and the synthetic process to the concentration of hormone has been well documented. It was first thought that their specific effects were mediated through this compound and in some cases this mechanism may apply. As a first approximation we have proposed that physiological changes and other agents which initiate changes in a tissue during cell division, development and other activities have two effects. They trigger a specific signal by means of binding to a specific carrier proteins and phosphorylation of histones turn on a particular synthetic process. They also somewhat non-specifically stimulate adenylyl cyclase and increase the rate of formation of 3',5' Cyclic AMP which turns on energy yielding processes such as the catabolic systems for fat and carbohydrate.

It should be noted that the 3',5' cyclic AMP dependent protein kinases which we have isolated from kidney phosphorylate glycogen synthetase, phosphorylase kinase as well as histones and casein. Our present interest in the regulation of glycogen synthetase and phosphorylase in lung and brain arose from the observation that the regulatory proteins also phosphorylate several different enzymes isolated from these tissues when nicotine is administered. Because of these observations we would like to undertake a study to examine the enzyme systems in lung and brain which are influenced by increased levels of 3',5' cyclic AMP.

2. SPECIFIC AIMS

The principal aims of this study will be (1) to determine the levels of 3',5' cyclic AMP in lung and brain of animals treated with nicotine and to compare these with control animals. (2) To determine the activities of adenylyl cyclase and phosphodiesterase after the administration of nicotine in these tissues (3) to isolate and compare the properties and activities of Cyclic AMP dependent protein kinases from the tissues of nicotine treated and control animals. To examine the ability of these "regulatory enzymes" to control the activity of glycolysis and lipolysis by influencing the activity of lipases, phosphorylase, glycogen synthetase and phosphofructokinase in brain and liver. (4) To determine the behavior and responsiveness of these enzyme systems to hormones in the tissues of these animals previously treated with nicotine.

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3. METHODS OF PROCEDURE

Different doses of nicotine ranging from 0.5-1.0 mg/kg of nicotine dissolved in saline will be injected intraperitoneally in rats, or doses ranging from 10-100 ug/kg intravenously, and the animals, together with controls receiving saline, will be sacrificed at various intervals after the administration of nicotine. In case of chronic studies, nicotine at doses ranging from 0.25-1.0 mg/kg will be given subcutaneously 4 times daily for at least 2-3 weeks and then the animals sacrificed. Tissues such as the heart, brain and lungs will be rapidly removed and homogenized in 0.25 M sucrose - 0.05 M Tris - 0.001 M EDTA. Activities of adenylyl cyclase, phosphodiesterase and protein kinases as well as the levels of 3',5' cyclic AMP in the nicotine-treated animals and controls will be determined by the methods previously developed in this laboratory.

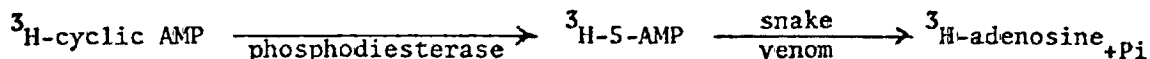
Sensitive enzymatic procedures based on the stimulation of glycogen synthetase kinase activity by 3',5' cyclic AMP have been used to assay the concentration of this compound in tissue extracts. Work in this laboratory has shown that a highly purified protein kinase isolated from kidney is dependent on 3',5' cyclic AMP for activity at the level of 10^{-8} M. This assay procedure measures the decrease in kidney glycogen synthetase activity or the transfer of 32 P from (λ^{32} P) ATP to glycogen synthetase. NaF and theophylline aid in the inhibition of enzymes which degrade the cyclic nucleotide. Controls in which small amounts of 3',5' cyclic AMP were added to the buffer used to prepare the extract showed that none of the nucleotide was lost between the time the tissue was extracted and before it was assayed. Other controls were run in order to ensure that the concentration of the cyclic nucleotide present in the extracts accurately reflected the amount which was present in the tissue. Labeled 3',5' cyclic AMP was added to the homogenizing medium and the nucleotide was reisolated by paper chromatography. The concentration of 3',5' cyclic AMP was then determined by isotope dilution and compared with the radioimmunoassay procedure. The assay procedure developed here has several advantages over existing methods for the measurement of cyclic AMP. The assay can measure levels of cyclic AMP in crude tissue extracts and as low as 10^{-13} moles of cyclic AMP. The method is also specific for cyclic AMP.

Adenylyl cyclase activity will be determined by incubating the crude extract or fresh membranes at 37° in a reaction mixture containing 40mM Tris-HCl (pH 8.0) 5mM MgCl₂, 1 mM cyclic AMP, 8mM phosphoenolpyruvate, 10 ug pyruvate kinase, 1 mM ATP and 0.2 microcuries of 3 H-ATP H-ATP (purified on a Dowex column AG50W-X8). At the end of the incubation time, 5 mM cyclic AMP will be added, and the tubes were boiled for 3 minutes. The solution is then passed through a Dowex 50-H⁺ column (3 x 65 mm).

The fraction containing cyclic AMP was collected and 0.2 ml ZnSO₄ (0.2M) and 0.1 ml of saturated Ba(OH)₂ were added. The pH was then adjusted to 7.0 and the supernatant liquid obtained after centrifugation was added to a scintillation fluid and counted.

Cyclic-AMP-Phosphodiesterase activity will be determined by an assay that is based on the following reactions.

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The entire reaction could be carried out in a plastic liquid scintillation vial. During a 10 min incubation at 37°, the substrate is converted to labeled ³H-5' AMP by the phosphodiesterase preparation and an excess of snake venom nucleotidase catalyses the conversion of ³H-AMP to tritiated adenosine. The reaction is stopped by the addition of an anion exchange resin that absorbs any unrelated substrate and consequently quenches its radioactivity. The reaction product ³H adenosine is not bound by the resin, thus allowing its radioactivity to be determined after the addition of the scintillation fluid.

Perfusion experiments will be carried out with lung and brain "in situ" by circulating or recirculating medium containing nicotine in the animals. The tissues will be rapidly removed at the end of perfusion and homogenized. The activities of adenyl cyclase, phosphodiesterase, protein kinase as well as the levels of 3', 5' cyclic AMP will be determined.

The effect of nicotine and several of its derivatives on the activity of specific glycolytic and lypolytic enzymes will be examined by adding them to a perfusate at a final concentration of 0.01 mM. Upon completion of the perfusion the lung and brain will be rapidly homogenized and the activity of various glycolytic enzymes, such as phosphorylase, glycogen synthetase, phosphofructokinase and pyruvic kinase, will be assayed. The results will be compared with those obtained in control experiments. Some of these enzymes as well as the cyclic 3',5' AMP dependent protein kinases and phosphoprotein phosphatases will be isolated from lung and brain. The effect of nicotine on these isolated enzyme systems will be examined and the results will be compared with data obtained from "in vivo" experiments.

4. SIGNIFICANCE OF RESEARCH

The experiments to be carried out in the course of this study will attempt to establish a more specific mechanism for the mode of action of nicotine and to more clearly define its effects on various mammalian tissues. These studies will also determine the nature of the interaction between 3',5' Cyclic AMP and nicotine. An understanding of the mechanism of action of nicotine either directly or through 3',5' cyclic AMP may help to explain some of the effects which are observed in lung and brain tissues when this compound is present.

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12&13. Biographical sketches of investigator and other professional personnel:

Dr. Joseph F. Mendicino- Birthdate-

REDACTED

B.S., Case Inst. of Tech., Cleveland Ohio, Chemistry, 1953.

Ph.D., Western Reserve Univ., Cleveland, Ohio, Biochemistry, 1958.

Postdoctoral, Inst. for Biochem., Invest., Buenos Aires, Biochemistry
1958-1960.

Postdoctoral, Western Reserve Univ., Cleveland, Ohio, Biochemistry 1960-1962.

Associate Professor; University of Georgia, Dept. of Biochemistry, 1968 to present.

Assistant Professor; Ohio State University; Department of Biochemistry, 1962-1968.

Postdoctoral Fellow; Western Reserve University (Dr. Harland Wood), 1960-1962.

Postdoctoral Fellow; Inst. for Biochemical Investigation, Buenos Aires, Argentina
(Drs. Luis Leloir) as a Research Fellow of National Foundation, 1958-1960.

The applicant went to the Department of Biochemistry of the School of Medicine at Western Reserve University for graduate training in biochemistry, receiving the Ph.D. degree in biochemistry with a physical chemistry minor in 1958. The graduate work was done with Dr. Merton Utter. Since 1958 he has been continuously engaged in biochemical research. From 1958 to 1960, as a postdoctoral research fellow of the National Foundation, studies were carried out in South America at the Institute for Biochemical Investigation in Buenos Aires, Argentina with Dr. Luis Leloir and from 1960 to 1962 at Western Reserve University with Dr. Harland Wood. The principal investigator is presently an Associate Professor in the Department of Biochemistry at the University of Georgia. A member of the American Society of Biological Chemists and American Chemical Society, Graduate Faculty and Institute of Comparative Medicine, University of Georgia.

Publications

1. Mendicino, J. and Muntz, J. A., The Activating Effect of Adenosine Triphosphate on Brain Adenylic Deaminase, J. Biol. Chem., 233, 178 (1958).
2. Mendicino, J., Effect of Borate on Alkali Catalyzed Isomerizations, J. Am. Chem. Soc., 82, 4975 (1960).
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Dr. Hussein Abou-Issa - Birthdate:

REDACTED

Univ. of Cairo, Egypt, B.S. (Hon.) 1951, Chemistry.
Univ. of Cairo, Egypt, Dip. Med. Sci., 1956, Biochem.-Bacteriol.
Univ. of Cairo, Egypt, M.S., 1960, Clinical Biochemistry
Univ. of Wisconsin, U.S.A., Ph.D., 1965, Biochemistry
Univ. of Georgia, U.S.A., Postdoc., 1970-Pres., Biochemistry

Professional Experience

REDACTED

Graduate Training - 1958-1960 M.S. degree in Biochemistry, University of Cairo.
1960-1965 at the University of Wisconsin, Madison, Wisconsin, Department of
Biochemistry. Received the Ph.D. degree in Biochemistry with Physiology as a
minor in 1965. The graduate work was done with Dr. W. W. Cleland.

Publications

Abou-Issa, H., Hickson, J., and Mendicino, J. F. Mechanism of Inactivation of
Swine Kidney and Rabbit Muscle Glycogen Synthetase by Soluble 3',5' Cyclic
AMP-Stimulated Protein Kinases J. Biol. Chem. (In Press).

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of Phosphofructokinase Fructose 1, 6-diphosphatase, glycogen synthetase and
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by kidney cortex mitochondria. Abstracts of the 162nd Am. Chem. Soc. Meeting,
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Abou-Issa, H. and Cleland, W. W. Enzymatic acylation of 1- α -glycerophosphate by
rat liver microsomes. Biochem. Biophys. Acta 176, 693, 1969.

Abou-Issa, H. and Abdel Kader, M. Mode of action of steroid hormones on
carbohydrate metabolism. J. Egypt Med. Assoc. 67, 1970.

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El-Mofty, A., Khattab, M., Abou-Issa, H. and Gaffar, A. Significance of Pyruvate and oxoglutarate in diabetes mellitus. U.A.R. J. of Chemistry 4, 55, 1961.

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#897 ROSAN

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

5
January 26, 1973

Grant Application No. 897

To: The committee comprising Drs. Jacobson, Loosli, and
Sommers

Subject: Robert C. Rosan, M.D., Cardinal Glennon Memorial Hospital
for Children, St. Louis, Missouri
New application No. 897
"Molecular Parameters of Lobular Regeneration and Function"

This memorandum supersedes that dated December 18, 1972
on Grant Application No. 647AR2 of this applicant.

Dr. Rosan has elected to waive his priority in com-
petition as renewal, and to submit the enclosed revised applica-
tion for a new grant.

May we suggest that you destroy the application sent
to you with our memorandum of December 18.

F.W.N.

FWN:wg
Encl.

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Comm.

Dr. Jacobson
Dr. Loosli
Dr. Sommers

CHRONIC PULMONARY DISEASE

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

No. 897

646-Act. 4/1/68

Ren. 4/1/69

4/1/70

646A-Act. 4/1/71

Ren. 4/1/72

Application For Research Grant

JAN 26 1973

Date: October 13, 1973

1. Name of Investigator(s): (include Title and Degrees) Robert C. Rosan, M.D.
Associate Professor of Pathology and Pediatrics, St. Louis University
Associate Pathologist, Cardinal Glennon Memorial Hospital for Children,

2. Institution &

Address: Cardinal Glennon Memorial Hospital for Children
1465 South Grand Boulevard
St. Louis, Missouri 63104

Checks to: Veljko Jellech
Chief Fund
Accountant

3. Short Title of Project:

Molecular parameters of lobular regeneration and function

4. Proposed Starting Date: April 1, 1973

5. Anticipated Duration of this Specific Study: Three years

6. Brief Description of Objectives or Specific Aims: In order to learn how chronic cellular injury in bronchioles leads to abnormal centrilobular repair, a novel quantitative model of irreversible lung injury will be evaluated through the correlation of molecular dissection with lung physiology, clinical and anatomical pathology, and roentgenography. Smoke is delivered by a Walton smoking machine.

1. The long-range goal: to develop predictive models of lung function after a specific irreversible injury to the small bronchioles.

2. The intermediate goal: to describe cellular alterations in the lung lobule that, after irreversible injury, lead to functional changes in ventilation.

3. The immediate goal: to describe selected processes of regeneration in the bronchiolar portion of the lung lobule after irreversible injury.

The selected processes include the cytoplasmic syntheses of these proteins, thought to typify the following events: glutamyl transpeptidase (cellular injury);¹ aspartic transcarbamylase (nuclear regeneration);² constitutive ribosomal protein (cytoplasmic regeneration);³ alkaline phosphatase isoenzyme (differentiated mucosal activity).⁴ These moities will be estimated in lung homogenates, histochemical stains, and peripheral blood. Lung function will be estimated primarily by a novel evaluation of pulmonary mixing.⁵ Clearance function will be estimated with the same tantalum powder used for roentgenographic study.⁶ After complete autopsies, lungs will be histologically evaluated by ordinal score technic⁷ and goblet cell counts.⁸ A special feature of the design is the attempted isolation of bronchiolar neurosecretory granules⁹ by molecular dissection, and the correlation of results with lobular growth, function, and repair as measured above.

7. Give a Brief Statement of your Working Hypothesis: Irreversible lobular injury is associated with measurable changes in cellular and molecular differentiation of the lobule. (Technological advances, including automated enzyme analysis, zonal ultra-centrifugation, advanced gas analysis systems, and multichannel physiological recording do permit a large-scale approach by an interdisciplinary team.)

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8. Details of Experimental Design and Procedures: (Attach Separate Pages)

This is a multidisciplinary design, in which the experimental animal inhales test material (oxygen or cigarette smoke), is followed by lung function studies (nitrogen washout and minute ventilation) and also by serum enzyme study (alkaline phosphatase isoenzymes, glutamyl transpeptidase, aspartic transcarbamylase) and hemograms; blood gases are measured in conjunction with the lung function studies. At autopsy, the lung tissue is itself studied for the molecular markers above, and as well for morphological variation which includes goblet cell counts and graded histology.

The essence of the design is that the animal is followed in the manner of a human patient. Multiple clinical, anatomical, radiological and biochemical variables are measured, and correlated with the progress of the lung disease.

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

All physical facilities required are within Cardinal Glennon Memorial Hospital for Children, except for the electron microscopy which is carried out by collaboration with a commercial consultation service (Sperry Rand).

10. Additional Requirements:

The need for one item on the budget, the cryostat microtome IEC #398 @ \$3,050 might be questioned at first glance; however, no such instrument is in our laboratory. Moreover, the surgical-autopsy facilities of Cardinal Glennon Hospital will be serving for a nearby 450 bed hospital while that hospital undergoes extensive remodeling. Under the circumstances, it would not be practical to add a research burden to the service load.

11. Biographical sketches of all principal and professional personnel (append)

12. List of publications: (Five most recent as pertinent) (append)

Asterisked in reference list.

1003538630

13. Budget: (1st year)

A. Salaries (Personnel by names)		% time	Amount			
Professional						
Robert C. Rosan, M.D.		10%	0.			
Technical	George Pounds, BS (Clinical biologist)	100%	9,200.			
	-----, BS (Physiologist)	100%	8,400.			
	-----, BS (Chemist)	100%	8,400.			
	Antoinette Cova, (Secretary)	25%	1,800.			
	-----, BS (X-ray technician)	10%	840.			
Total Fringe on above			2,680.			
B. Consumable Supplies (list by categories)		SUB-TOTAL	31,320.			
Guinea pig care and maintenance, 10¢/d x 40 pigs x 365 d			1,460.			
Miscellaneous Glassware			500.			
Miscellaneous Chemicals			750.			
Radionuclear supplies (for aspartyl transcarbamylase)			800.			
		Sub-Total	3,510.			
C. Other Expenses (itemize)						
Pro-rated permanent equipment maintenance, 50%			750.			
Electron microscope consultant (Sperry Rand, 20 pictures x \$55.00			1,100.			
Computer time (Hewlett-Packard 9800) \$3.20/hr x 80 hr			256.			
Travel (To electron microscope consultant, Washington, DC x 2) . . .			369.			
		Sub-Total	2,475.			
D. Permanent Equipment (itemize)						
Fluorescence-phase equipment to convert existent research microscope, Wilde N-20, Wilde Cat. #259-022, 243-395, 268-054, 268-022, 250-390, and 105-844			6,090.			
Fluorescence equipment to convert existent Coleman 129 spectrophotometer to fluorescence, Coleman Cat. #139-0251, 139-0481			1,775.			
Cryostat microtome, Damon Co. IEC #398			3,050.			
Ultracentrifuge rotor, Damon Co. IEC #494			800.			
		SUB-TOTAL	11,715.			
E. Overhead (15% of A + B + C)		TOTAL	49,020.			
E. Overhead (15% of A + B + C)		Total \$5,595.00				
Estimated Future Requirements:		TOTAL	54,615.			
	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	32,760	3,685	2,599	0	4,099	43,143
Year 3	34,358	3,369	2,728	0	4,304	45,299

*Includes 5% annual cost-of living increases.

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature *Robert C. Rosan, M.D.*
 Director of Project Robert C. Rosan, M.D.
 Telephone
 Signature *Patricia M. Helms-Lewis*
 Business Officer of the Institution
 Telephone

1003538631

Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current

Title of Project

Source

Amount

Duration

NHLI RFP 72-25 Chronic pulmonary disease and
smoking (animal model)

National Heart and Lung Institute
(Contract)

\$78,000.00

one year

ending: None

1003538632

8. Details of Experimental Design and Procedures:

A. INTRODUCTION

For the past three years, The Council for Tobacco Research - U.S.A. (CTR) has supported our study of molecular and cellular alterations associated with growth, differentiation, and regeneration of injured human and animal lung. The results and significance of this work will be summarized below. As a direct recognition of these studies, we recently received a renewable competitive National Heart and Lung Institute (NIH) contract (RF #72-25) for \$78,000 which establishes both the core team and the basic equipment to conduct a wide spectrum of experimental studies on irreversible bronchiolar injury. In this CTR proposal, we plan to use the new NIH experience in leap-frog fashion, that is, to apply the fully matured interdisciplinary approach which NIH now nourishes to the next phase of our CTR research. Furthermore, we expect that a "multiplier effect" may stimulate serendipitous advances when NIH and CTR groups are at work in experimental areas which are physically, intellectually, scientifically, and clinically contiguous portions of a dynamic biomedical landscape.

B. THE UNIT LOBULE HYPOTHESIS

The most important issue from previous CTR husbandry of our work is a new theory of lung growth, function, and repair, which I term the unit lobule hypothesis (Fig. 1).

In brief, the theory states that the lung functions as an assemblage of coordinated units, called unit lobules,¹⁰ rather than as a mass organ.

These lobules contain, in their apical regions, the regulatory mechanisms for lobular gas flow, perfusion of the microcirculation, and significantly, modulation of the milieu interieur of the parenchymal lung tissue.^{10,11}

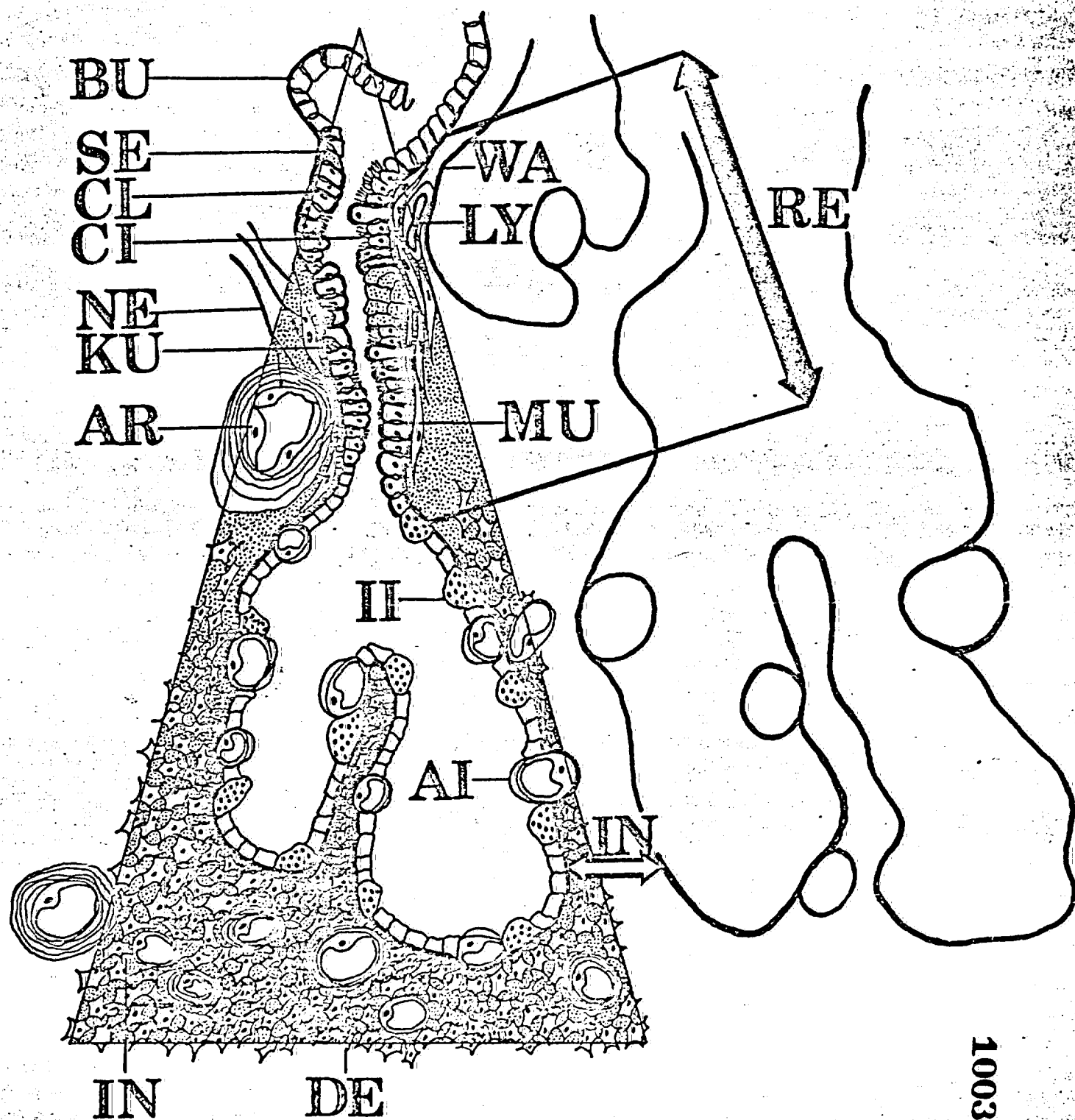
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FIGURE 1 FETAL/PREATURE/NEONATAL UNIT LOBULE

- BU Budding of bronchiole into primitive air sac
- SE Secretory mucosa associated with tubular secretion/resorption
- CL Clara cell
- CI Ciliated cell
- NE Nerve
- KU Kultschitsky (neurosecretory) cell
- AR Muscular arteriole
- IN Intersaccular septum of bulky mesenchyme
- DE Deep microcirculation of potential intrapulmonary shunts
- AI Air-blood barrier
- II Type II cell
- MU Bronchiolar smooth muscle
- LY Lymphatic in typical parabronchiolar position
- WA Alveolar wall (juxtaposition of alveole and bronchiole)
- RE Regulatory area of lobule containing active tissue

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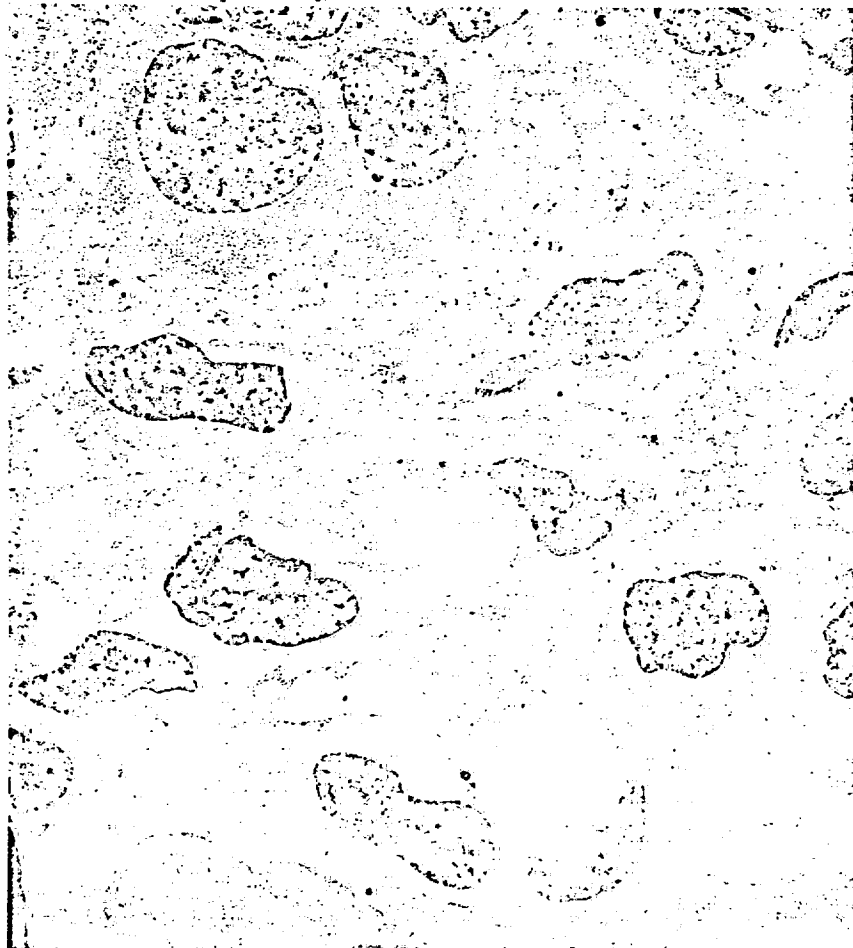
FIG. 1



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Fig. 2

The critical "regulatory region" of Fig. 1, as seen in a 600 g liveborn infant. Note the excellent development of arterioles, and the close association of bronchiolar smooth muscle with mucosa above and arteriole below. The loose, mesenchymal character of the peribronchiolar tissue is well seen. We believe this morphology helps explain the great compliance (see Fig. 28) and great susceptibility of neonatal bronchioles with respect to peribronchiolar edema.



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The Unit Lobule Hypothesis

POSTULATION:

The basic functional module of the lung is the unit lobule, which consists of an apical regulatory region and a basal passive region.

COROLLARIES:

1. Bronchiolar compliance is a significant mechanical feature of apical lobular function which is subjected to continuous active regulation.
2. Homeostasis of the interstitium, including that of the basal passive region, is modulated by the apical regulatory region.
3. Effective function of the lobule is vested in the active process whereby the component functions are integrated.
4. Mass lung action is the product of integrated lobular function.
5. Regeneration of the injured lobule in part recapitulates its development.

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Thus, the unit lobule consists of: (a) an apical regulatory region associated with neurogenic, muscular, endothelial, mucosal and lymphoid differentiation, and (b) a basal passive region, the vascular tissue lattice of the intra-alveolar septae (or in immature lungs, the intersaccular septae)¹² associated with a distinctive organoid, the air-blood barrier, a surfactant system and a macrophage system. That this hypothesis has been received with interest in the academic community may be judged by the evidence that we have been asked to contribute chapters to three books for three different audiences within one year,^{13,14,15} and have had numerous requests for Fig. 1.

The first five corollaries implied by the unit lobule hypothesis follow. An obligative corollary and one of the particular novelties of this hypothesis is this: the bronchioles are among the most compliant portions of the lobule. This notion is in sharp relief to the established surfactant dogma of Clements, Avery, and others¹⁶ but it is supported by the authoritative voices of Auerbach¹⁷ and Thurlbeck,¹⁸ by data of post-mortem babies from Strang's group,¹⁹ by experimental and clinical studies of oxygen toxicity by ourselves^{20,21} and others,²² and by direct observations of Towers.²³ For the present, the chorus of these observations seems to constitute a rising crescendo. Our most recent observations on positive end-expiratory ventilation of babies, strongly supports these arguments (Fig. 23)²⁴ It is the active regulation of bronchiolar compliance which helps regulate lobular gas flow.

In a second corollary, we now propose that active airway control achieved by neuromuscular and mucosal mechanisms pari passu helps regulate the milieu interieur of parenchymal lung tissue through its effects on lymph flow in parabronchiolar lymph channels. Indeed, we believe that parenchymal homeostasis and airway mechanics are intimately interlocked. The advantage of

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the well-known close association of peri-arteriolar and peri-bronchiolar smooth muscle with lymphatic channels is clear; but we believe that the bronchiolar mucosa itself actively participates in the exchange of fluids between the lumens of airways and the lobular interstitium. It was Clara who originally proposed the analogy of bronchiolar mucosae to renal tubular epithelia,²⁵ and none of the morphologic evidence of recent ultrastructural advances can contradict his idea.¹¹ Rather, the morphological adaptation of Clara cells (Fig. 3) suggests to us a specialization for (a) regulation of intercellular fluid exchange (b) intracellular synthesis of surface coat components (c) elaboration and translocation of other materials, e.g. those which subserve clearance and compliance, as well as other functions.¹¹ A third corollary states that the function of the lobule is vested in the integration of its components. This contrasts again to the monolithic outlook of surfactant dogma. More importantly, and from the perspective of experimental design, from this corollary flows the claim that no single lobular parameter can be manipulated experimentally without inducing change in other of the functional components of the lobule. In effect, this corollary is the converse of a commonsense pathologists' observation: a wide variety of pulmonary insults produces a stereotyped monotony of responses. To paraphrase, a small number of final common pathways is implicit in the functional and molecular anatomy of the unit lobule. Thus, the lobule is a system, to be analyzed by systems analysis, and thus susceptible of computer modeling. However, a steady-state system must maintain its own equilibrium through feed-back control; hence, this corollary predicts that important lobular feedback controls exist.

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A fourth corollary states that, because the unit lobule is the fundamental module of functional lung anatomy, mass lung action is secured through the coordination of these units. Thus, the whole lung is also a system, and not the sum of its atomistic parts. Rather, the system is an expression of the activities of numerous unit lobules which are not necessarily completely synchronized nor completely equivalent. To demonstrate that this is indeed the mode by which the normal lung functions, one should be able to explain the well-known physiological data of normally encountered ventilation:perfusion inequities. We interpret certain observations of Lauweryns,²⁶ Towers,^{23,27} Shanklin,²² and others, and our own data on oxygen toxicity^{20,21,28} that indeed it is normal for some lobules to be at work or in one phase of ventilation, while others are at rest or in a different phase.

The fifth and shortest corollary states that regeneration of the injured unit lobule in part recapitulates its normal ontogeny. It is this corollary for which the least data exist, and it is to this area of ignorance that our proposed activity is heavily directed.

For the purpose of this proposal, the main hypothesis and the five corollaries are tabulated in abbreviated form (Fig. 3). We anticipate that further experience may suggest modifications or even rejection of some of the above ideas, and count that as a beneficial result of an organized theoretical framework. On the other hand, we also anticipate that additional corollaries will suggest their presence when the experimental data are generated.

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C. EXPERIMENTAL DESIGN

(1) Summary:

Guinea pigs under close clinical observation are challenged with controlled doses of oxygen or cigarette smoke delivered by a Walton smoking machine. Periodically, nitrogen wash-out is analyzed as a function of pulmonary mixing, minute ventilation is also evaluated, the peripheral blood is analyzed by means of a hemogram, the blood gases and pH are analyzed, and three test enzymes are estimated (see below). Prior to sacrifice, a bronchogram is arranged by tantalum particle technic and bronchiolar clearance is estimated by a follow-up radiograph. At autopsy, the lung is analyzed under three headings: lung homogenates are examined after sucrose gradient zonal ultracentrifugation for their ribosomes, neurosecretory granules, and enzyme content of g-glutamyl transpeptidase, alkaline phosphatase, and aspartic transcarbamylase. The frozen lung is examined for its content of neurosecretory cells by fluorescence microscopy, and for alkaline phosphatase by classic histochemistry; fixed lung is diagnosed, its lesions scored by a multimodal ordinal technic, and its population of mucus cells and tantalum-containing macrophages estimated. The apical or regulatory region of the bronchiole is further evaluated through collaboration with an experienced electron microscopist.

The above multidisciplinary design provides a description of some regenerative events in the bronchiole after irreversible injury, which is our first goal (item 6, p. 1).

It also supplies clues to cellular alterations associated

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with physiological alterations, our second goal, and some inferences for a predictive model of lung function after specific injury, our third goal. The design proposes no new methods, but rather an amalgamation of technics currently in use in two different research studies in our laboratory. Thus, all projected work loads are based on our own experience, particularly with advanced high-volume instrumentation.

(2) Details of experimental design:

a. Animals:

Purebred newborn strain II guinea pigs are from pedigree stock maintained and bred by us within our laboratory, in stainless steel Hoeltge cages, on Ralston Purina guinea pig chow ad lib. Temperature and humidity are continuously recorded by a Rustrak #255 dual channel strip chart; light, temperature, and human egress are controlled.

Individual cages of test and control animals are rotated on the rack at regular intervals according to a randomization procedure, in order to equalize all environmental and ecological effects, such as differential distribution of light, temperature, odor and noise. All animals are routinely subjected to bacteriological surveillance.

Newborn animals to be admitted to the protocol must weigh more than 80 g. and be healthy at birth. They must be the issue of dams at least four months old.

Eligibility of dams for mating is judged by observation of the vaginal plug. Complete pedigree records are kept. Matings are scheduled.

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b. Exposure to pulmonary irritant:

Oxygen exposure is carried out in a Labconco hood in flowing 100% or 60% oxygen according to our published method (7). The dose of oxygen is computed as hr%, viz. hours of exposure multiplied by the concentration of oxygen administered (29).

Smoke exposure is carried out according to a progressive scale of smoking, by which the daily frequency of exposure is increased, but the exposure period itself is constant (Fig. 4). Kentucky Standard Cigarettes, previously secured in large lots and stored under refrigeration, are smoked in a Walton positive pressure smoking machine secured under the auspices of Dr. J. H. Kreisher. The dose of cigarette smoke is calculated by the chlorinated hydrocarbon method of Alex Spears (to be published; personal communication J. H. Kreisher) with a chlorobiphenyl tracer compound. Controls "smoke" unlit cigarettes. The smoking schedule (Fig. 4) is tentative and subject to revision in accord with current experience. Cigarette dose is computed five ways: the total number of hours exposed, total length of cigarette smoked, total weight of cigarette smoked, and mean post-exposure hemoglobin: carboxyhemoglobin ratio estimated from spectrophotometric shift in the oxyhemoglobin Soret bands, and by Spear's tracer method.

In the standard smoking mode, the lit, measured, weighed cigarette is marked 2 mm from the hot tip and placed in the smoking machine and timed and observed. We anticipate development of Skinner-box experiments during the current NIH contract which will make acceptance of cigarettes a voluntary response of the animals but this is not a necessary part of the experimental design.

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FIG. 4

SCHEDULE OF EXPOSURE AND DOSE FOR
PULMONARY IRRITANTS: OXYGEN AND TOBACCO

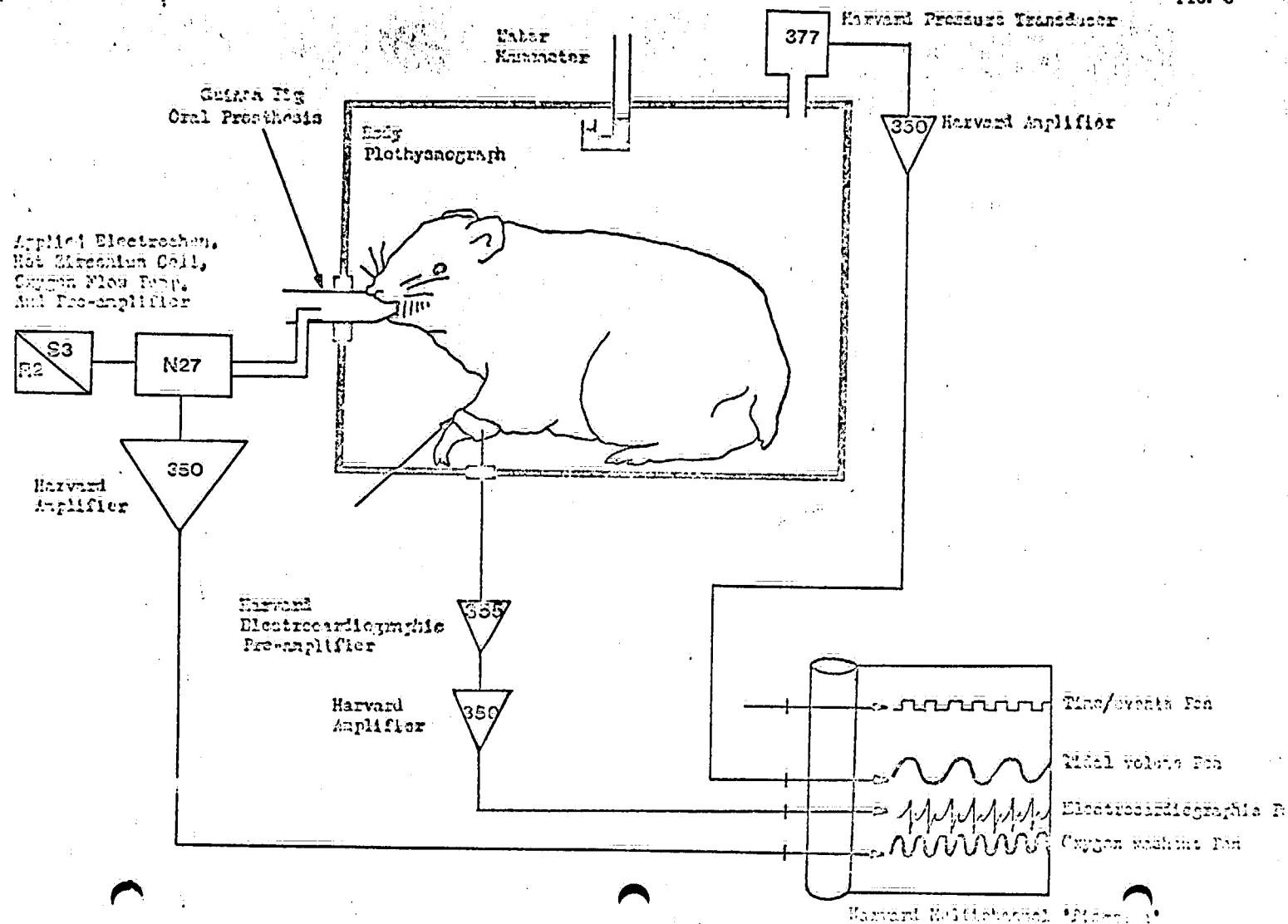
AGENT	STRENGTH	WEEKS OF EXPOSURE		
		0 - 4	4 - 8	8 - 16
OXYGEN	HIGH DOSE (100%)	CONTINUOUS	--	--
	LOW DOSE (60%)	CONTINUOUS	CONTINUOUS	CONTINUOUS
CIGARETTE*	HIGH DOSE	1*	2	4
	LOW DOSE	1*	1	1

*1 numerical dose equals 3 mm cigarette smoked in 1 exposure

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Diagram of Guinea Pig In Plethysmograph for Physiological Lung Function Studies

FIG. 8



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c. Methods and materials: (FIGS. 9 & 10)

1. Clinical evaluation:

- (a) Appearance: daily recorded check of coat, mouth, chest, abdomen, excreta; includes timed period for handling.
- (b) Activity: daily recorded check of activity, posture, gait, and response to standard non-conditioned stress.
- (c) Weight: Ohaus animal balance (± 0.1 g.) daily.
- (d) Rectal temperature: Yellow Springs Instrument amplifier #43TA, and probe #402 ($\pm 0.1^\circ$ C) thrice weekly.
- (e) Hemogram: red cell count, hemoglobin, hematocrit, computed indices, and white cell count by Coulter S; differential smear by Romanowsky stain, weekly.

2. Serum chemistry (biweekly):

- (a) Alkaline phosphatase: isoenzyme estimation from gel acrylamide electrophoresis, modified from the method of Gluck by us (30,31,32), and performed for total serum enzyme by automated replicate technic in Abbott ABA 100A analyzer. This instrument is explained and depicted in Fig. 11 A & B, and the machine method has been adapted by us from a "Sigma" kit.
- (b) G-Glutamyl transpeptidase: total enzyme estimation by the p-nitroanilide method of Orlowski (33,34) according to the formula: 2 g-glutamyl-p-nitroanilide + g-glutamyl transpeptidase \longrightarrow (g-glutamyl)(g-glutamyl)-p-nitroanilide + p-nitroaniline; method is modified by us for replicate estimation on the Abbott 100A analyzer (35). A "Dade" kit is used.
- (c) Aspartic transcarbamylase: total enzyme method of Porter, Modebe, and Stark (36), as modified by Normal Kretchmer and Nicholas Hoogenraad (personal communication). In this method,

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SCHEDULES OF TYPES OF IN VIVO EXAMINATION

FIG. 9

EXAMINATION	TESTS PERFORMED	WHEN PERFORMED				AT TERMINATION
		FIRST DAY OF LIFE	THRICE WEEKLY	BI-WEEKLY		
				IN PLETHYSMOGRAPH	NOT IN PLETHYSMOGRAPH	
PHYSIOLOGICAL LUNG FUNCTION EVALUATION	PO ₂ PCO ₂ pH Minute ventilation Nitrogen washout			+		
CLINICAL EVALUATION	Appearance Weight Ventilatory rate Pulse Temperature	+	+			+
CLINICAL LABORATORY EVALUATION OF BLOOD	Hemoglobin/hematocrit Red cell indices Leucocyte count Differential smear Carbon monoxide Aspartyl transcarbamylase Glutamyl transpeptidase Isozymal alkaline phosphatase	+			+	
TISSUE PATHOLOGY EVALUATION*	Subgross giant section Histologic grades 3 enzymes (above) Neurosecretory granules Goblet cell count Tantalum macrophage count					+

*Note: electron microscopy on selected specimens only, based on tissue pathology evaluation.

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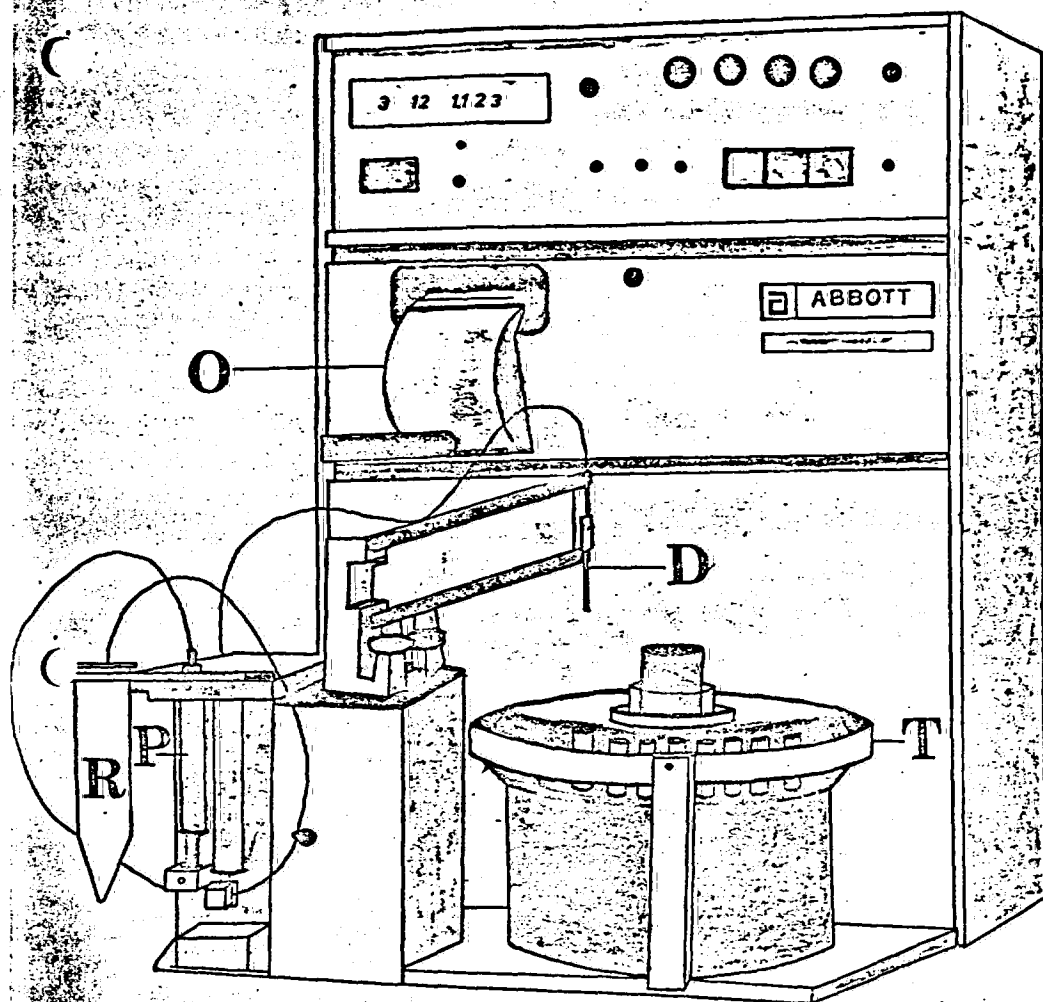
FLOW DIAGRAM OF BI-WEEKLY PHYSIOLOGICAL LUNG FUNCTION TESTS

WITH ALLOCATIONS OF EQUIPMENT AND PERSONNEL

TEST DATA	STEADY STATE 10 min	COLLECT DATA PERIOD	INHALES 100% O ₂ 5 min	COLLECT DATA PERIOD	STEADY STATE 10 min	METHOD AND EQUIPMENT	PERSONNEL
SINGLE LEAD ELECTROCARDIOGRAM	+	+	+	+	+	Electronic amplification of pulse, respiration, minute ventilation; Harvard Apparatus Co. amplifiers, transducers, multichannel chart.	Technician #1 (physiologist)
VENTILATORY RATE	+	+	+	+	+		
MINUTE VENTILATION		+					
NITROGEN WASHOUT (pulmonary mixing)		+		+		High temperature elec- trochemical cell plus above; Applied Electro- chemistry Co. instrument.	
P _A O ₂		+		+		Micro-electrode digital mobile blood gas station; Corning 165C analyzer.	Technician #2 (clinical chemist)
P _A CO ₂		+		+			
pH		+		+			
CARBON MONOXIDE		+				Scanning 2-wavelength Spectrophotometry; Perkin-Elmer 124	

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FIG. 11 A



- O = Output on tape of true kinetic data
 D = Dipping needle on arm to introduce reaction mixture and transfer samples
 R = Reservoir for reaction mixture
 P = Pump syringe for dispensing reaction mixture automatically
 T = Turntable with sample tubes in outer ring, cuvettes in middle ring (not shown) and 2-wavelength spectrometer in center (not shown)

This is the only two-wavelength automated enzyme analysis system available, and has been in use for the last four months. The particular advantage is the much greater accuracy and precision over a much wider range (see Fig. 11 B). See reference #35.

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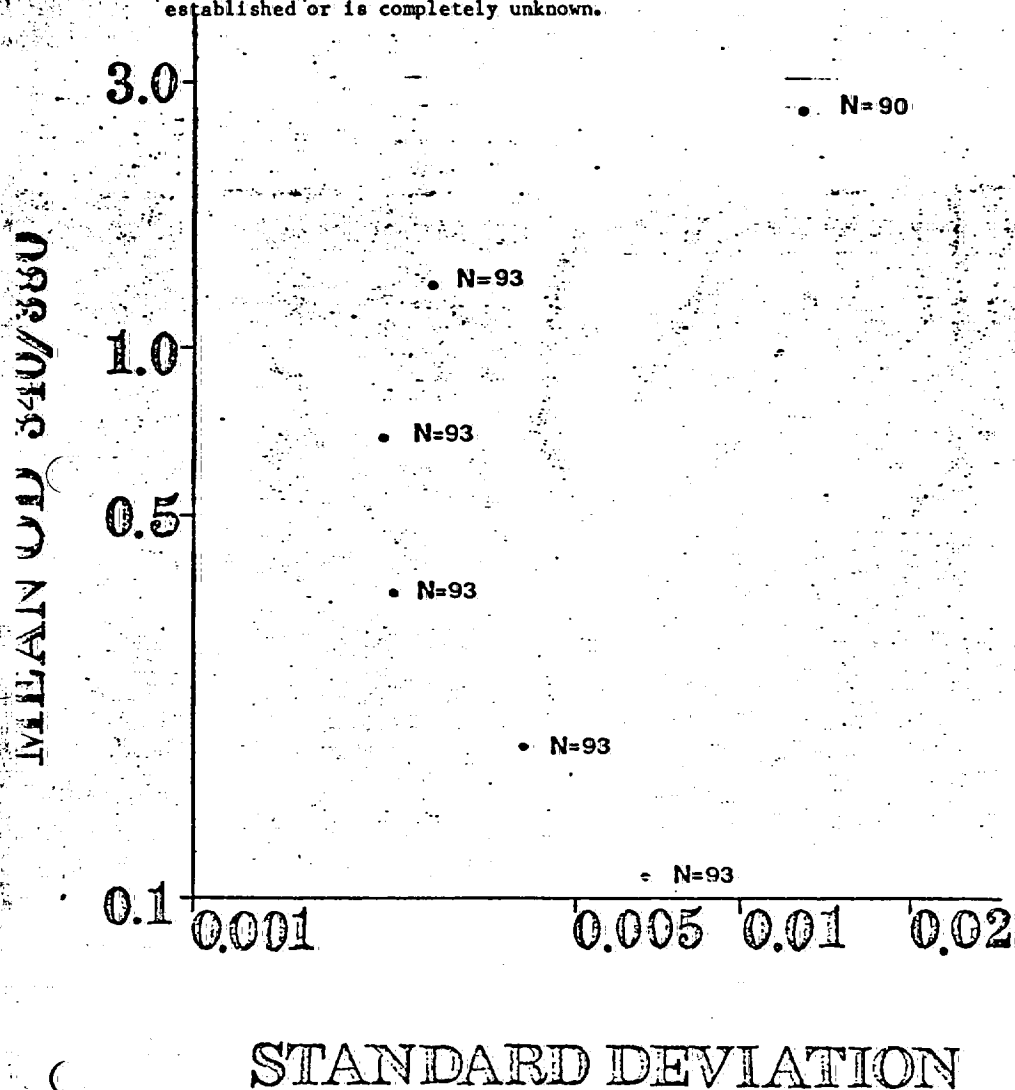
NADH

FIG. 11 B

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SAMPLES INTRODUCED MANUALLY

Two-wavelength enzyme measuring technic. Standard deviations are acceptable even when enzymatic activity is measured at very high optical density. This makes the instrument especially useful for research investigations in which the order-of-magnitude concentration of the sought for enzyme has not been established or is completely unknown.



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^{14}C -L-Aspartate is the substrate; the product of enzymatic activity, ^{14}C -carbamylaspartate, is generated by interaction with carbamyl phosphate. Then, the product is isolated on Dowex 50 ion exchange resin (H^+ form) and eluted with buffer:

$$\text{Car-P} + ^{14}\text{C-Asp} + \text{aspartic transcarbamylase} \longrightarrow \text{Car}(^{14}\text{C-Asp}) + \text{P.}$$

The reaction is carried out for 15 minutes at 37°C after a 1-minute preincubation, and ended by precipitating enzyme with trichloroacetic acid. We count it on a Nuclear Chicago Unilux II, with computations carried out on a Hewlett Packard 9800 micro-computer.

(To our knowledge, this is the first proposal to evaluate the presence of aspartic transcarbamylase in serum except for our own current NIH contract work. The importance is that, unlike all common clinical tests of serum enzyme activity, that for aspartic transcarbamylase evaluates the presence of an enzyme specific and obligate for the synthesis of DNA during mammalian cell replication. Thus, presence of aspartic transcarbamylase in serum in increased amounts is a definitive marker for regenerative nuclear activity, whereas all other enzymes in clinical analysis are markers for injured or regenerating cytoplasmic systems, or increased cell membrane permeability.)

(d) Blood gases:

These are established from capillary blood samples, by the use of a Corning 165C blood gas station. This machine computes PO_2 , PCO_2 , (HCO_3^-) , and pH from 100 μl samples, which we plan

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on a biweekly schedule. The use of "arterialized" capillary gases has received extensive comment in human literature on neonates; briefly, the method is valid but in establishing relative rather than absolute values. The machine and technic are in routine use in our current studies, and normal values have been established for strain II guinea pigs, by us.

(3) Pulmonary function studies (biweekly):

Under the current NIH contract, a complete ventilation station has been constructed and is in use and it is this which we shall utilize.

The station is based upon a body plethysmograph designed by us specifically for studying guinea pigs, together with a novel mouthpiece which we design and produce from casts of the guinea pig's oral cavity (Fig. 5,6,7)

Thus, the animal is snugly confined within the plethysmograph by a bulky restraint, and the tightly fitted mouthpiece protrudes through a gas-tight port. A Harvard Biograph #2120 5-channel strip chart simultaneously records pulse, respirations, tidal exchange, and pulmonary mixing, as explained below. (The fifth channel is a time-and-events pen.)

- a. Pulse is recorded by a single lead electrocardiograph.
- b. Respirations are recorded with a pneumotachygraph.
- c. Tidal exchange is measured with a Harvard #377 pressure transducer.
- d. Pulmonary mixing:

Nitrogen washout is measured by a novel method. As usual (37), the animal is subjected to 100% oxygen for 5 minutes. Then, by multiple-breath open-circuit technic, the change in P_{AN_2} , after cessation of 100% oxygen, is analyzed by means of a rapid-action hot zirconium cell.

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However, the innovation is that the cell actually measures $P_{A}O_2$ which, in the absence of marked CO_2 retention, is an accurate reciprocal measure of $P_{A}N_2$ when the vapor tension of water is constant: $P_{A}O_2 \sim 760 - (P_{A}H_2O + P_{A}CO_2)$ when $P_{A}N_2 \rightarrow 0$. The method makes use of the fact that fast action (c. 35 msec) oxygen cells of quite low dead space (c. 200 μ l) are available in clinically useful form, e.g. Allied Electrochemistry #S-3/R-1/N-22M (Fig. 8).

(The basic assumptions for use are tenable: by the time CO_2 retention occurs, the physiological demonstration of uneven ventilation is redundant in these experiments; and the alveolar vapor tension of water is rarely subject to significant change in any event. The frequency response of the Harvard Biograph, in excess of 100 Hz, is sufficient to handle the graphic output; and there is also available a Hewlett Packard 7400 A X-Y recorder for more linear output at a slewing speed in excess of 250 cm/sec. This method is in routine use in our NIH contract work.) (Fig. 27)

e. Minute ventilation:

This is computed by calibration of the displacement of the Harvard pressure transducer used with the body plethysmograph (Fig. 8) and then integration of the resultant strip chart readings with respect to the areas under the curve. Whether the test animal is in steady state may in part be confirmed from the simultaneous electrocardiograph and breathing frequency records. The period of observation is such that, excluding sighs, all the observed results are statistically linear (flat) with time.

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(4) Chest Roentgenography:

- a. The technic is essentially that we have published before (7) except that it is now possible to get improved resolution with the use of Siemens tube with an 0.9 mm focal spot, instead of the former 2 mm size. The beam is used unfiltered at 850 ma, 40 KV, 1/125 sec, at a precisely constant distance of approximately 65 cm; Kodak type AA film or the equivalent is used with par-speed screens, and developed in a high speed Picker automatic hot processor. The animals are immobilized in a plastic rack during roentgenography.

b. Tantalum powder bronchography:

The technic is that of Nadel, Wolfe and Graf (6); the basis is the radio-opacity of aerosolized tantalum powder, combined with its availability in tightly controlled particle size, and its relatively banal non-toxic behavior in all human and animal experiments to date. This technic is also part of our current N.I.H. Contract routine. In use, accurately measured weights of dry tantalum powder of 10 μ particle size are dispensed into a small chamber equipped with a recirculating Muffin fan.

The test animal is placed within the body plethysmograph (Fig.8) and connected by means of his oral prosthesis to the tantalum powder chamber. He breathes powder by closed circuit technic for precisely 1.0 minutes during which time his minute ventilation and tidal volume is recorded. Six hours (\pm 1) and 48 hours (\pm 6) later, he is roentgenographed by the described technic. Directly thereafter, at a recorded time, he is sacrificed and autopsied.

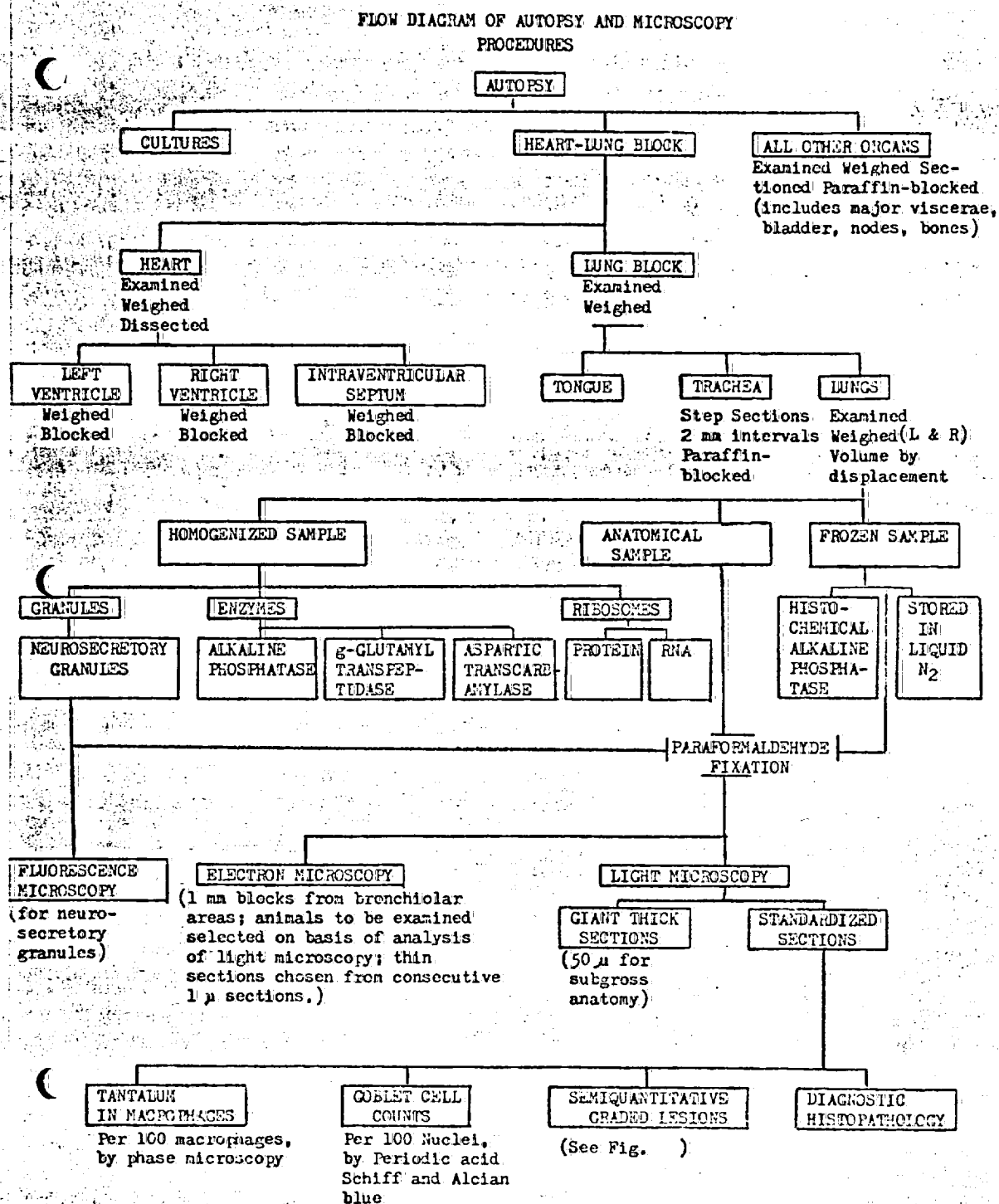
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(5) Autopsy procedures:

- a. General necropsy: The details are shown in Fig. 12-14. In brief, all organs except the brain are dissected and examined; the major viscera are weighed, and specific notations are entered as shown. The animals are despatched by a blow to the skull; the lungs are rapidly dissected, weighed, and volume measured by displacement, so that material for electron microscopy and molecular dissection is available within 30 seconds and well preserved. After, there is a more leisurely gross examination of the remainder of the specimens.
- b. Heart: The heart is weighed and examined, and the anterior right ventricular wall, posterior left ventricular wall, and intraventricular septum are separately dissected and weighed. The objective is to evaluate possible right ventricular hypertrophy in the light of functional and histological lung lesions (Fig. 12).
- c. Lungs: The weight and displacement are recorded (above). Then, three types of samples are recovered from three standard loci, as follows. A 1-mm block of the full lung, from apex to base, and through the hilum is removed in the lateral plane. This block is the source for all anatomical material; the remainder, or the bulk of the lung tissue, is for molecular dissection (see below). Of this block, the right middle lobe is arbitrarily the source of all material for electron microscopy, alkaline phosphatase histochemistry, and secondary fluorescence of neurosecretory granules; the last two evaluations are performed with material frozen at -170°C . Details of these methods are given below. In addition, 5 μ and 50 μ sections are prepared from regular paraffin-embedded material of upper and lower lobes.

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FIG. 12



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FIG. 13 A

AUTOPSY INFORMATION CHECK LIST (PART 1)

REPORT # _____

ANIMAL IDENTIFICATION: _____

FIXATIVE: HCHO: _____

GENEALOGY: _____

DATE: _____

PARA: _____

PICTURES: _____

TIME: _____

GLUT: _____

FROZEN: _____

PROSECTOR: _____

OTHER: _____

AGE: _____ days

COAT: _____

SEX: M F

HEAD: _____

WEIGHT: _____ g

BREASTS: _____

LENGTH: _____

UMBILICUS: _____

NOSE-RUMP: _____ cm

GENITALIA: _____

NOSE-TOES: _____ cm

LIMBS: _____

CHEST: _____ cm

PLEURAL CAVITY:

PERICARDIAL CAVITY:

MEDIASTINUM:

ABDOMEN:

RETROPERITONEUM:

REMARKS:

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FIG. 13 B

DETAILED SYSTEMIC EXAMINATION (PART 2)

REPORT # _____

SYSTEM	ORGAN	WEIGHT	DIAGNOSIS		OTHER/ADDITIONAL
			GROSS	MICROSCOPIC	
VENTILATORY	TONGUE	-	-	+	
	TRACHEA	-	-	+	PAS- Alcian Blue
	CARINA	-	-	+	PAS- Alcian Blue
	LUNG	R	+	See Fig.	PAS- Alcian Blue
		L			Elastic Van Giesen
CARDIO- VASCULAR	HEART	R. Vent			
		L. Vent	+	+	
		I-V Septum			
	GREAT VESSELS	-	-	+	Elastic Van Giesen
URINARY	KIDNEY	+	+	+	
	BLADDER	-	+	+	
LYMPHO- RETICULAR	SPLEEN	+	+	+	Giemsa
	NODES	-	+	+	
	THYMUS	+	+	+	
	PEYER PATCHES	-	-	+	
	BONE MARROW	-	-	+	Giemsa
GASTRO- INTESTINAL	ESOPHAGUS	-	-	+	
	STOMACH	-	-	+	
	DUODENUM	-	-	+	Single Specimen
	JEJUNUM	-	-	+	
	ILEUM-CECUM	-	-	+	
	COLON	-	-	+	Single Specimen
	LIVER	-	-	+	
ENDOCRINE- GONADAL	PANCREAS	-	-	+	
	PITUITARY	-	-	+	PAS- Orange G
	THYROID	-	-	+	
	PARATHYROID	-	-	+	
	ADRENAL	+	-	+	
	GONADS	+	-	+	
	UTERUS/ VESICLES	+	-	+	

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CATEGORY	GRADE 0 (NORMAL)	GRADE I (REACTION)	GRADE II (NECROSIS)	GRADE III (REGENERATION)	GRADE IV (CHRONICITY)
BRONCHIOLAR MUCOSA	normal	> 25% of cells necrobiotic or exfoliating	> 50% of cells necrotic	> 25% of cells atypical or metaplastic	plus hyperplasia
ARTERIOLES	normal	edema of muscle	muscular proliferation	plus intimal proliferation	plus periarteriolar infiltrate
LYMPHATICS	normal	dilated	severely dilated	perilymphatic infiltration	perilymphatic fibrosis
GOBLET CELLS	normal	25% increase	50% increase	100% increase	100% increase
HYALINE MEMBRANES AND/OR EDEMA	absent	10-50% of air sacs	50-100% of air sacs	plus 10-50% of bronchioles	plus 50-100% of bronchioles
INFLATION PATTERN	normal	> 25% alveolar atelectasis	> 25% lobular or lobar atelectasis	plus lobular distention	plus lobular emphysema
ALVEOLAR INTERSTITIUM	normal	edema	focal fibroplasia	diffuse fibroplasia	plus fibrosis
ALVEOLAR INFLAMMATORY REACTION	neutrophiles absent	5% air spaces with neutrophiles	33% air spaces with neutrophiles	foci of pus	neutrophiles and plasma cells
	macrophages	5% air spaces with free macro- phages	33% air spaces with free macro- phages	macrophage plugs in airways	macrophage pro- liferation filling lobule
HEMORRHAGE	absent	10-50% of alveoli	50-100% of alveoli	plus extension into bronchioles	plus interstitial in 2 or more major septae

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The 5 μ sections are evaluated double-blind in four ways: routine histopathological diagnosis, ordinal grades of specific lesions (Fig. 12), estimation and location of tantalum-containing macrophages, based on phase microscopy; and a sensitive fluorescent periodic acid Schiff and Alcian blue routine, previously used by us (38) and adapted to the small mucosal cells of normal guinea pigs. Details of this methodology are also given below.

1. Electron microscopy: This is carried out on 1 mm blocks which have previously been identified as primarily bronchiolar in fresh material under the dissecting microscope at necropsy according to our published methods (29). The pH is controlled in order to maximize standardization of observation for neurosecretory granules (39); after we embed it in Epon, the material is stained by osmification at controlled pH (40). The objective of the microscopist is to provide 15 publishable pictures documenting the cellular changes and alterations in neurosecretory granules when irreversible injury is compared to the control. Therefore, "thin" sections will be selected by decisions reached after the visualization of juxtaposed "thick" sections in phase, light, and fluorescence (41) microscopy.
2. Alkaline phosphatase histochemistry: This is carried on frozen sections post-fixed in paraformaldehyde, with the classic naphthol AS-MX coupling technic at pH 8.6 in the presence of $MnCl_2$ (42); reagents are from a 'Sigma' kit, and the counterstain is Mayer's hematoxylin. The objective of this test is to determine the cellular sources of enzyme isolated on the ultracentrifuge (below) as well as to provide information about the differentiation of mucosal and endothelial cells before, during, and after irreversible injury. This is also the enzyme of our choice

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with which to begin future ultrastructural studies of specific microsomal protein production after irreversible injury, inasmuch as the cyto-enzyme technic has been frequently published from other laboratories, and we have a well-tried method available (43) which has been modified for optimal performance (Bengt Robertson, personal communication).

3. Neurosecretory granules: The active material in pulmonary Kultschitsky cells is not known, but is universally assumed to be enterochromaffin and neurosecretory in nature; these cells are included by Pearse in the so-called APUD family of amine and kinin secreting cells (44). It is well accepted that active granules fluoresce upon reaction with small aldehyde molecules; typically, dopamine, noradrenaline, or 5-hydroxytryptamine is reacted with formaldehyde, paraformaldehyde, or glutaraldehyde. The reaction, not well understood, has been discussed by Corrodi (45). We have chose a modification worked out in the laboratory of our close collaborator, J. M. Iauweryns (46).

At necropsy, 1-mm lung blocks from standard areas (see Fig. 12) are plunged into isopentane at at -170°C in liquid nitrogen, and then freeze-dried with a Virtis cold trap and a Cenco vacuum pump. The dry material is reacted with formaldehyde generated from paraformaldehyde at a relative humidity of $60\% \pm 10$, by the use of concentrated sulfuric acid, and exposure of the sections to the vapor in a closed chamber is accomplished for one hour at 80°C . This develops fluorescence of dopamine, 5-hydroxytryptamine, and noradrenaline; an additional two hours develops adrenaline (47). The sections are mounted in polyester wax and flouresced with an Osram HB 200 mercury lamp at about 404 nm with a BG 12 filter, and observed above 520 nm under darkfield conditions with barrier filters Schott GG-9 and OG-1. Results are checked by phase microscopy and by silver staining (argyrophilia); controls are checked by the borohydride method (45).

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4. Ordinal grades for semiquantitative evaluation of lesions:

This method is based on our previous observations of oxygen-induced rat bronchiolar lesions (48), which have been confirmed by similar ordinal grading of guinea pig, (7) mouse (49), and human infant (21) lungs after oxygen intoxication. The table of ordinal grades is shown (Fig. 14); in the method, hematoxylin-eosin stained paraffin sections of entire lung in the mid-lateral plane then are evaluated double-blind.

5. Goblet cell counts:

The major innovation is that the goblet cells are counted by a fluorescent rather than a visible periodic acid Schiff reaction, by the substitution of acriflavine for the more common Schiff's fuchsin; the remainder of the reaction is classical. (5). We have previously used this technic in paraffin and plastic embedded specimens. (5).

The reason for the fluorescence application is that bronchiolar goblet cells of guinea pigs are often quite difficult to visualize in ordinary light technic. In order to count goblet cells per 100 nuclei, a nuclear stain of Alcian blue at pH 2.8 is useful, since it not only stains nuclei quite well by fluorescence (R. C. Rosan, unpublished) but gives additional clues to the type of glycoprotein in bronchiolar glycocalyx and in the mucus-producing apparatus (3). The fluorescence equipment and optical conditions are the same as for neurosecretory granules (above). By double blind technic, all goblet cells in the entire section of whole lung in the mid-lateral plane are counted.

6. Tantalum particle macrophage counts:

The same sections used above are re-examined for tantalum-containing macrophages by phase microscopy. The total number of macrophages

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is also counted, and a ratio established. In the event the macrophages are too numerous to count, i.e. over 1000 present in an entire section, sample areas will be examined by a randomization technic. The macrophage counts will also be related to the total number of airways per section. The tantalum content of each cell counted will be estimated in four ordinal grades: (0) no observable particles; (1) less than 5 particles (2) less than 50 particles (3) more than 50 particles.

7. Ordinary histopathologic diagnosis:

This is the usual diagnostic technic by which pneumonitis, chronic fibrosis, and other possible lesions may be classified. However, we do not propose to use or investigate the term emphysema in this proposal; instead, purely descriptive terms will be used (e.g. centrolobular bronchiolar atrophy, alveolar septal atrophy, etc.).

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There exist tabulated times and gradient volumes for a B-14 zonal rotor, which we shall adapt and use; they give figures for *Euglena* spp. and *E. coli* ribosomes, which are quite close in physical characteristics to human ones (54).

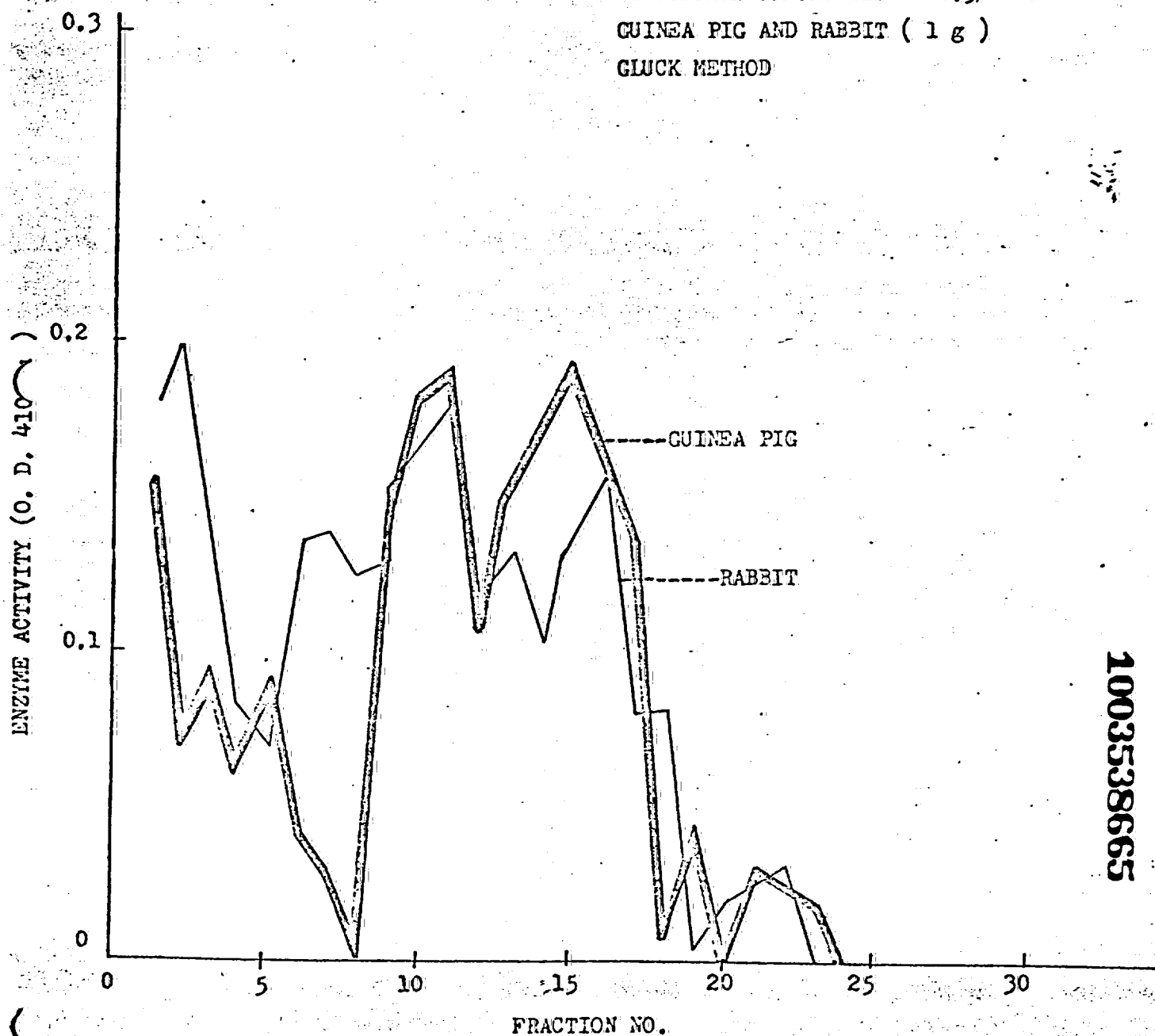
The non-linear gradients are formed with a pre-programmed ISCO dialagrad #380 pump, and the rotor is unloaded with additional 2M sucrose solution, so that the effluent is directed through a 2-channel recorder (see a. l. above) to a fractionator as before. However, the resolution is greatly increased, approximately 3-8x by zonal technic. Also, this rotor effects a great savings in labor due to its much higher capacity. This permits up to 10 animal experiments to be pooled (and thus averaged) in a single batch.

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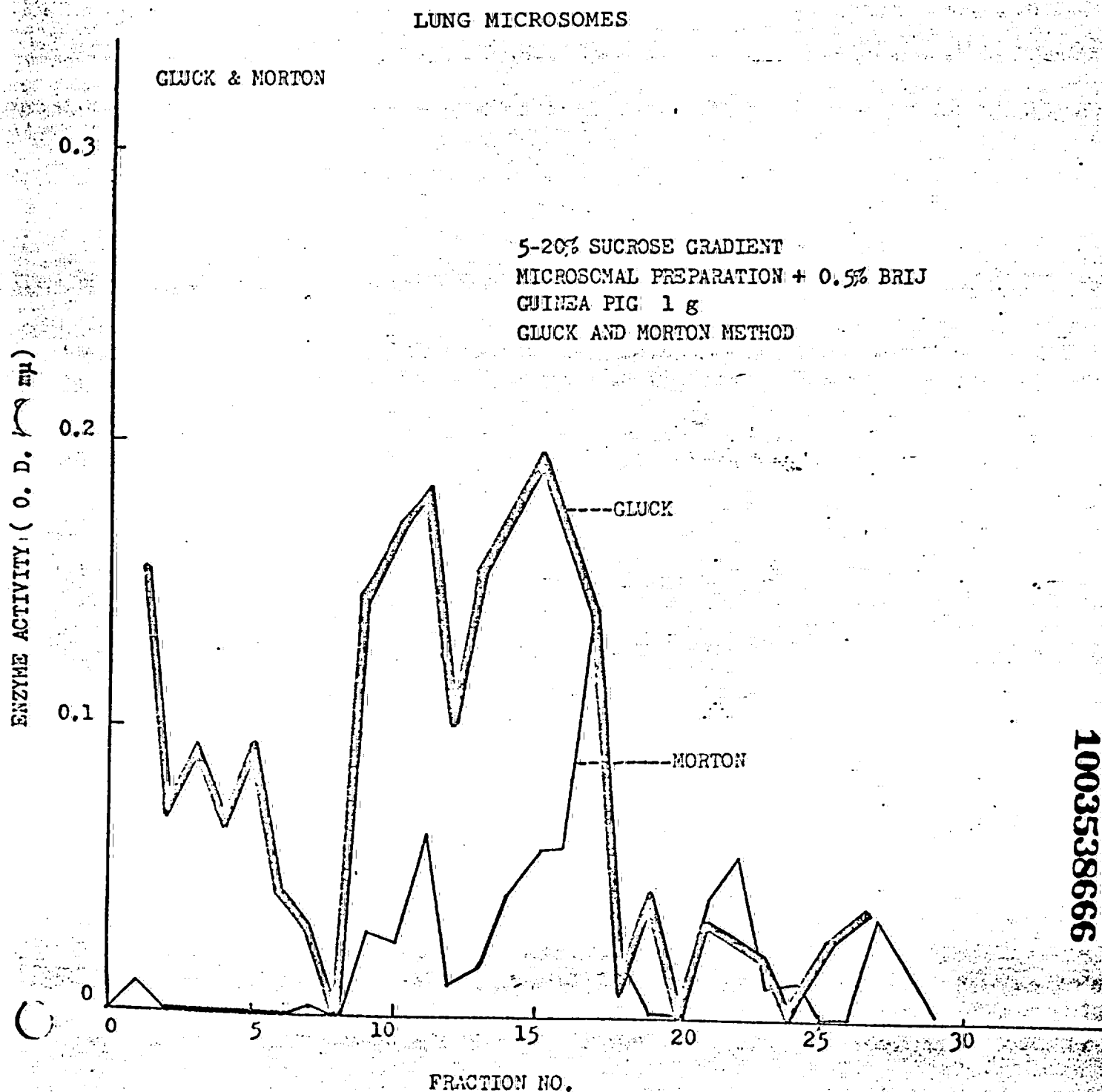
LUNG MICROSOMES

RABBIT AND GUINEA PIG

5-20% SUCROSE GRADIENT
MICROSOMAL PREPARATION + 0.5% BRIJ
GUINEA PIG AND RABBIT (1 g)
GLUCK METHOD



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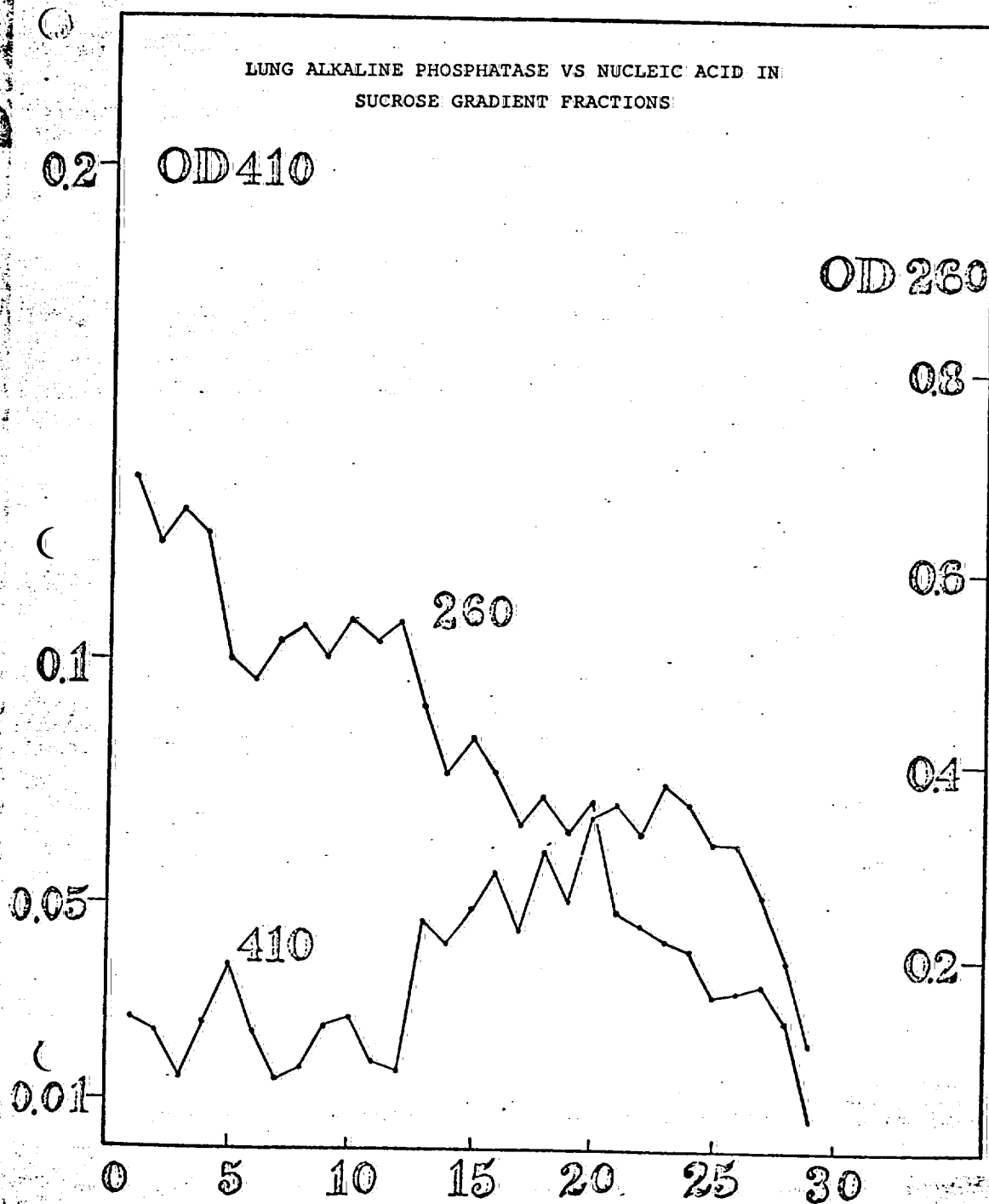


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UNBORN (CANALICULAR)
MEAN WEIGHT ~ 40 GRAMS

FIG. 17

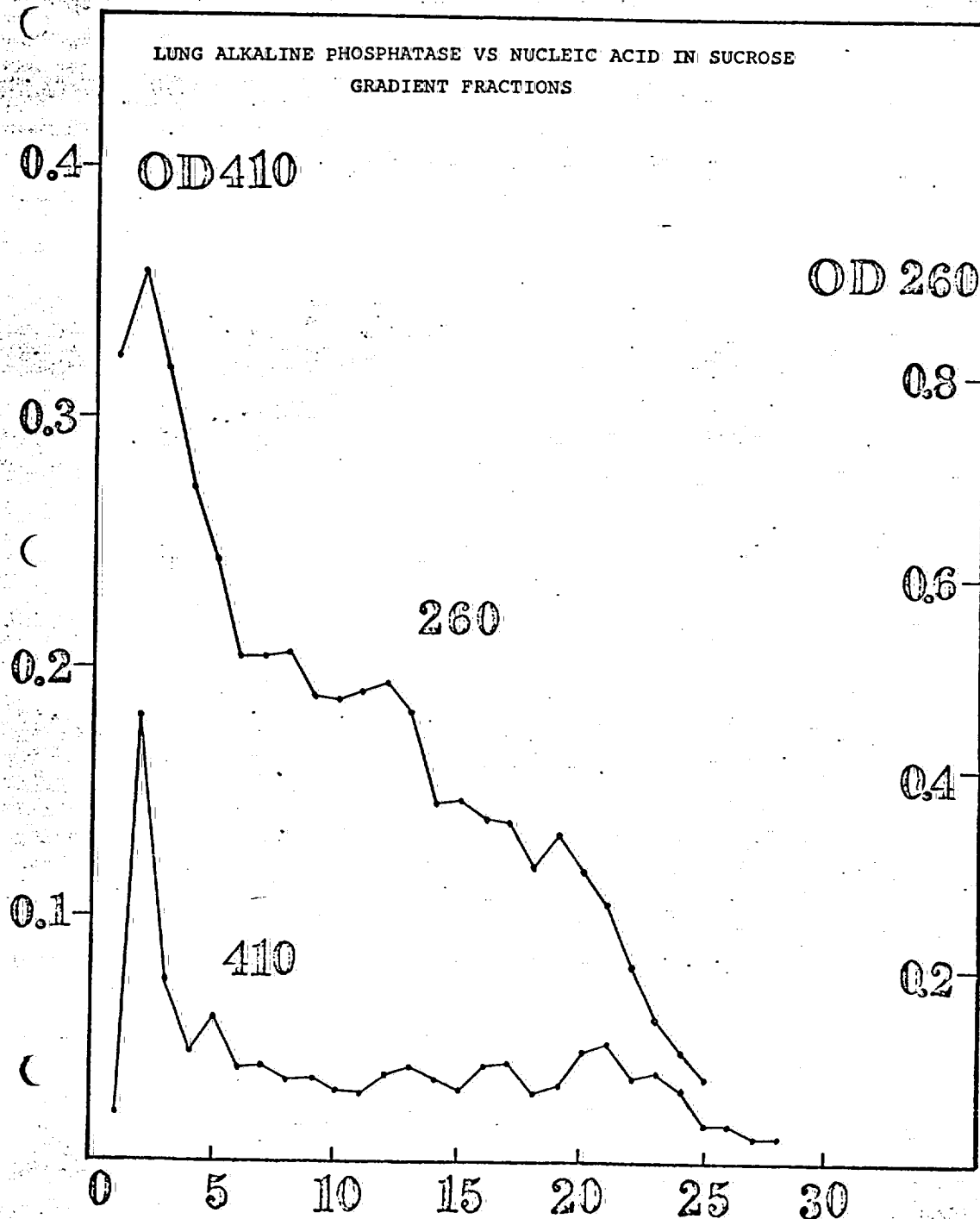
(42)



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NEWBORN (SACCULAR)
MEAN WEIGHT ~ 55 GRAMS

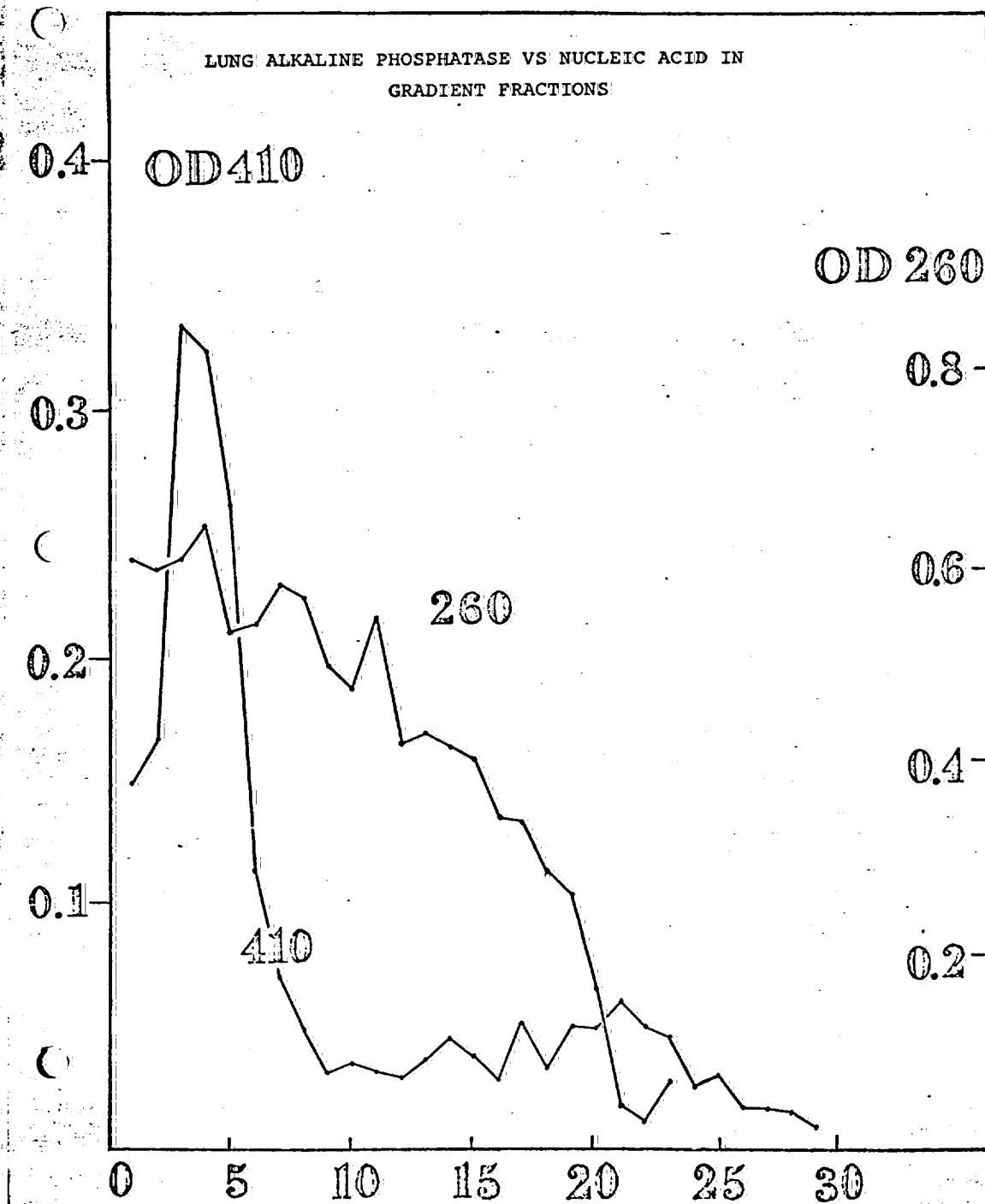
FIG. 18



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ADULT (ALVEOLAR)
MEAN WEIGHT ~ 835 GRAMS

FIG. 19 (44)

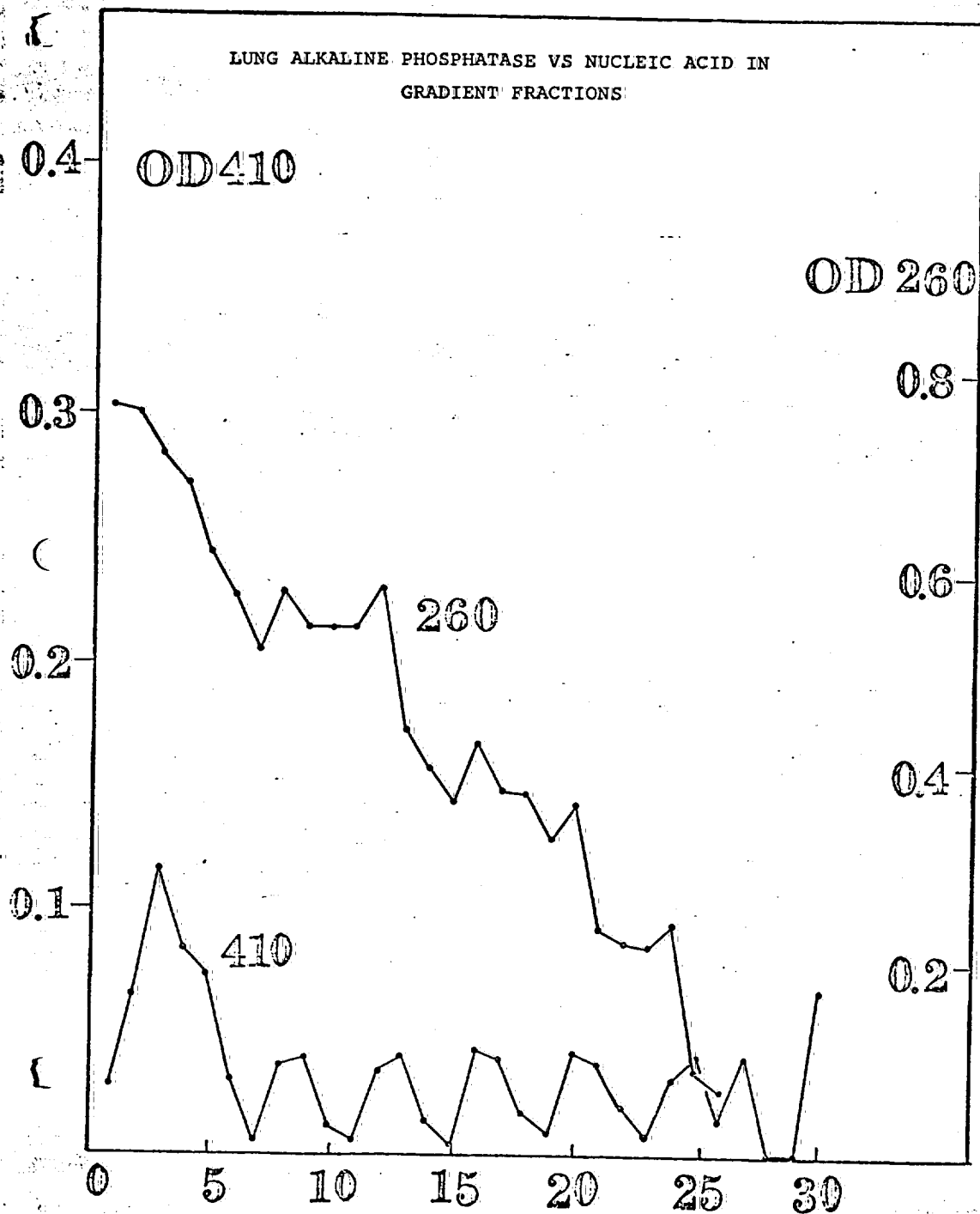


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JUVENILE (ALVEOLAR)
MEAN WEIGHT ~ 500 GRAMS

FIG. 29

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(d) Lung homogenates:

All procedures are based on the reduction of bulk lung tissue to a single homogenate (see item (5) c. above), which is then subjected to ultracentrifugation. The high speed fraction, which contains microsomes, ribosomes (3,50), small vesicles, and neurosecretory granules (51), is then analyzed by appropriate study of fractions prepared from sucrose gradients.

1. General method of fraction preparation Fig. 29.

- a. The method and its modifications have been previously published from our laboratory (3,50,52,53). (This is not the method of choice, but is the backup method for zonal ultracentrifugation, see below. However, numerous mechanical and instrumental misadventures (none of them due to the technic of our laboratory) have kept our zonal rotor out of service approximately 78% of working hours in the past 18 months. Therefore, while we prefer to carry out the experiment outlined on the zonal rotor, and while numerous other investigators have achieved similar or more demanding analytical separations, we are no longer sanguine that a complete study may be planned about the zonal method alone.)

The lungs are flushed through the pulmonary artery in situ with Ringer's solution (pH 7.4, 0°C), immediately resected, and minced with razor blades. The mince is forced through an hydraulic press (Carver Company) at 2000 psi, and the resultant "brei" is strained through cotton gauze to remove gross lipid. The "brei" is homogenized in a Teflon pestle in buffer-detergent (0.0015M MgCl₂, 0.01M KCl, 0.001M 'Tris', 0.25M sucrose, 0.5% 'Brij-35', pH 8.0 at 0°C) with six 30-sec passes of the pestle at 500 rpm. A supernate is obtained by slow-speed centrifugation, 10,000 g x 15 min

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(International Equipment Co. #HT, #856 angle head), in the above buffer at 4°C. This is repeated, but at 35,000 g. The mitochondrial-free, nuclear-free, opalescent material obtained we term "ribo".

The above "ribo" is formed into a pellet in the above buffer (less 'Brij-35') and centrifuged at 105,000 g x 2 hrs at 4°C (International Equipment Co. #B-60, rotor #488). The pellet is gently resuspended in buffer and the procedure repeated. The final pellet is placed on a 5-20% sucrose gradient, non-linear in the 14-17% region as programmed from an ISCO Dialagrad #380 pump, and centrifuged in plastic tubes in the above rotor for 3 hrs at 45,000 rpm over a 2M sucrose cushion. The tubes are pierced from the bottom; the gradients are forced out by a flow of 2M sucrose from a Harvard pump, the flow is monitored by tandem ultraviolet recording sensors at 254 and 280 nm by an ISCO UA-2 #580 dual beam monitor, and about 30 fractions are cut by an ISCO #272 drop collector and carousel.

The methods given above are in fact a precis of those which we have successfully used and published previously. The fractions are then evaluated for their ribosomal content, ribosomal protein and ribonucleic acid, aspartic transcarbamylase, alkaline phosphatase, glutamyl transpeptidase and neurosecretory granules as outlined below.

b. Zonal ultracentrifugation:

The method for zonal preparation of particles is the technic of choice although, as explained above, its availability depends

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upon mechanical function of an instrument which has worked well for others in the past but not us.

The preparation termed "ribo" (see a. 1. above) is used to load the zonal rotor (IEC #B-29), with the usual buffer system (see above). The sample is visibly marked through the introduction of blue Dextran, and injected at the core between a 5% sucrose layer beneath a 42 ml layer of 0-3% sucrose above.

The method of centrifugation is the new equivolumetric technic of Pollack and Price (54). In brief, it is required that there be a constant C such that:

$$C = \frac{r^2}{r \cdot n} (d_p - r \cdot d_m)$$

r = radial distance of particle from center
 n = viscosity of sucrose buffer
 d_p = density of particle (ribosome = 1.41)
 d_m = density of sucrose buffer

They assume that a gradient can be designed such that a particle zone of zero radial thickness will migrate through equal volumes of gradient in equal increments of time. They do in fact demonstrate that for ribosomes loads of 5 mg or less, a particle zone of 10 ml or essentially negligible thickness may be obtained, which would for example give a resolution sufficient to separate 15-unit polysomes from 14-unit polysomes and 18S particles from 20S.

1. Enzymatic activity: the crude fractions, without further dialysis, concentration, nor clarification, which we term "cru-f", are tested for enzymatic activity with respect to alkaline phosphatase isoenzyme, g-glutamyl transpeptidase, and aspartic transcarbamylase. Since the methods used are exactly those outlined for serum enzymes under item (c. 2. Methods and materials) above, the technology will not be further discussed here.

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2. Total protein: Aliquots of "cru-f" fractions are dialyzed exhaustively in a benzoylated membrane bag against distilled water and then tested for total protein by Lowry's method. When the results warrant, i.e. more than 20 ug protein, and if time permits, the protein is subjected to acrylamide gel electrophoresis in 7% gel at pH 8.4, for isoenzyme stains.

3. Neurosecretory granules:

(a) Wet chemical evaluation of bioamines from neurosecretory granules (51,56)

The technic is essentially that of Cottrell, i.e. sucrose gradient fractions are examined for bioamine activity by a fluorescence method (51). The fractionated homogenate (above) is extracted with 1:5 1N formic acid : acetone; the extract is dried in a stream of nitrogen and then briskly agitated in one volume of 0.01N HCl-saturated isobutanol for 5 min. Then, there is a second extraction with a 2X volume of low-fluorescence heptane during another 5-min. agitation period; the organic and aqueous phases then are separated at 1000 g x 5 min. The material of interest is placed on a 6 x 85 mm column of ion exchange resin (Amberlite CG-50, #2, 200-400 mesh) previously equilibrated with 0.2M phosphate buffer at pH 6.1, and is eluted with 1N HCl. General fluorometric conditions for analyses of the eluate are:

SUBSTRATE	PROCEDURE	FLUORESCENCE nm	
		ACTIVATION	EMISSION
5-hydroxytryptamine	5N HCl	300	545
Dopamine	Hydroxyindole (alkaline sulfate- acetic acid)	325	390
Noradenaline	Hydroxyindole (alkaline sulfate- acetic acid)	385	485

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There are no innovations in this account from that published.

In general, this method is considered to be a quantitative and specialized one, used as indicated to follow up upon the results of the screening test below. This method is precise at the nanomole range \pm 10% approximately.

(b) Thin layer chromatography of bioamines from neurosecretory granules

An acid butanol extract, prepared as above, is spotted on thin layer plates of microcrystalline cellulose (Merck 'Avicel', without fluorescent indicator) and developed in the ascending mode with 60 : 20 : 20 isobutanol : methanol : 1N formic acid for four hours; the plates are dried at 56°C and redeveloped at right angles with 60 : 35 : 5 chloroform : methanol : 1N ammonium hydroxide for two hours. (47). The developed plate is reacted with paraformaldehyde vapors and fluoresced at 360 nm in order to locate the three major substrates. Alternately, all of the spots are developed with diazotized p-nitroaniline. This method is capable of resolving sixteen biologically significant amines, including many of the chief congeners of dopamine, 5-hydroxytryptamine, and noradrenaline, and including those three substrates themselves, and is sensitive in the nanomole range.

(c) Fluorescence of neurosecretory granules

The Falck-Hillarp method (45), of condensation with formaldehyde vapors to produce a fluorescent product in situ, is the method of choice and one we have observed in use (46). A recent minor modification of the method is selected (57). Cryostat microtome sections are prepared in 7% gelatin. Isopentane quenched material, at -170°C, is brought to -120°C, cut, and then dessicated at -60°C for three days at 0.001 torr with the aid of a Cenco pump and a Virtis dry-ice trap. It is then cut at 5 μ , and examined for fluorescence as in (5) a. 5. above.

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4. Ribosome analyses:

These analyses depend upon the availability of the zonal ultracentrifugation head. In order for the ribosomes to be isolated by fractionation of the sucrose gradient as in item (5) c. above, and in accord with our published work (3,50,52), and marked with 'Blue' Sephadex, molecular weight 2,000,000.

The 83.5S peak, recognized from 254/280 nm dual channel records of the fractionated sucrose gradient (above) is formed into a pellet at 105,000 g x 5 hr in buffered sucrose in a swinging bucket head. The material is re-purified in this manner until the sample shows a 260/235 nm absorption ratio over 1.50, or it is rejected entirely.

The pellet is resuspended in a chelating buffer (0.02M KHCO_3 , 0.001M HPO_4^{2-} buffer at pH 7.3, 0.03M KCl, 0.004M ethylenediaminetetraacetate (EDTA)) to separate ribosomal subunits, and placed over a 10-20% sucrose non-linear gradient consisting of the above buffer plus 0.2M MgCl_2 , less EDTA. This is spun at 110,000 g x 5 hr in an IEC zonal rotor #B-30. The 50S subunits (52), identified by the previously described ISCO dual channel recorder, are isolated by fractionation of the gradient, and dissociated in the above phosphate buffer plus 0.5M LiCl, 0.2M MgCl_2 , and 6M urea.

This material is purified by zonal centrifugation at 150,000 g x 15 hr and dialyzed against 0.033M sodium acetate in 6M urea. The contents of the bag are concentrated when the bag is dehydrated in a bed of Sephadex G-200, and the product is electrophoresed on 12% acrylamide gel at pH 4.3 in the presence of 6M urea. The resultant bands are stained with Coomassie blue and read in our special integrating microphotometer (52).

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(3) Significance of work:

a. Defects in current experimental designs:

For years, we and many other anatomical pathologists have failed to develop models of lung injury which measure or correlate with actual physiological function. Equally, lung physiologists have avoided coming to grips with the alleged discrepancies between the histological appearance and micro-function of the injured lung. Indeed, micro-function --lobular function-- has hardly been studied by any discipline. And molecular biologists, excepting the few who deal with the specific problems of the type II cell, have conspicuously avoided molecular dissection and the cell biology of the lung.

Where does that leave us today? We stand on the verge of considerable advances in understanding how several important organs operate as systems: the brain, the kidney, the heart, several endocrine glands, and the liver -- but not the lung.

In the list of tissues examined in dozens of monthly journals for this or that enzyme, organelle, or macromolecular product, or its regulatory actions, the lung is usually conspicuous by its absence. We want to remedy that situation. For several years, and mostly with the aid of The Council for Tobacco Research support, we have been attempting to develop a broadly based laboratory devoted to the systems analysis as well as the operational analysis of injured lung.

b. Systems analysis:

By systems analysis, we mean the study of the lung as an assemblage of modules and components, the interactions of which determine

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the function and fate of the injured organ. The key word is "interaction". In our model, we view the lung as a system of units, of which the incidental function is ventilation of the blood stream. Thus, the lung has the same needs for tissue homeostasis, for cellular nutrition, and for molecular regulatory mechanisms as the other organs in the body. To study the lung as organized around the air within it has no more rationale than to the study the heart as organized around the blood within it, or the kidney about the urine within it. To study the lung as a system, therefore, implies the capability and the confidence to relate its different tissue and cellular functions one to another -- or more briefly, to study the interactions. Thus, the multidisciplinary approach is intrinsic.

c. Operational analysis:

As to operational analysis, by this we mean a study of what the lung does. Again, we believe that the movement of blood and gases for ventilatory purposes is only one of many functions of the lung. But the first thing the lung does, to complement the above systems approach, is to keep itself alive and well as an organ. Therefore, from both the systems and the operational approach, we require that the lung be studied as tissue, with special recognition of its self-regulatory functions, its cellular biology, its macromolecular control mechanisms.

How do \dot{V}/\dot{Q} defects relate to secretory molecular changes? How can defective tissue homeostasis result in a loss of bronchiolar compliance? Will bioamine-induced arteriolar tone modulate

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the pumping of lymph? How does Clara cell function change with chronic obstructive airways disease? Is it inappropriate to speak of endexpiratory bronchiolar "collapse" when the role of the actively contractile bronchiolar muscle is not considered? What does the Kultschitsky cell do? Is there a calculus of lung function, i.e. does the lung function as a mass organ or as an integrated collection of subunits? What is the effect of destroying the integration function, if it exists, as opposed to destroying discrete anatomical targets? Are there valid computer models for self-regulating lung functions? The answers to these questions are perforce interdisciplinary, and cannot be studied outside of a systems framework, in an operational manner.

d. Immediate significance:

Thus, a major immediate significance of our work lies in its innovative approach to the lung. It would not be revolutionary in the study of, say, transport of salt by frog bladder, or, for example, memory by brain tissue. But applied to lung, the approach is most uncommon.

In an operational and systems context of a real laboratory, the revolution turns out to be unexpectedly simple in concept. It asks that we study sick animals in much the same way that we study sick humans. We would not presume to follow a patient seriously ill with bronchitis without complete blood counts, clinical chemistry and blood gas studies, periodic measurements of pulmonary function, and chest roentgenographs.

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We propose to follow sick animals the same way. To this we add the possibilities of a more thorough autopsy study because with animals, unlike with humans, we can secure necropsied tissue with almost no autolytic artefacts. So the "new" approach is as conservative as it is revolutionary. It is nothing more than the application of the principles of sound clinical judgment to the evaluation of a sick patient. It is in itself an exploration of what is clinically relevant in experimental airways obstruction, an attempt to get input for human disease on a rapid basis.

e. Long range significance:

The ultimate significance has the possibility to be quite large.

First, if the approach is successful, it would change the style of some other researchers from monodisciplinary to multidisciplinary.

Second, it would systematically apply a tremendous amount of clinical knowledge and equipment -- multiphasic screening comes to mind -- to the management of experiments in which the animals are supposed to be models of sick humans; thus, a larger yield of directly useful clinical insights might come to light.

Third, and possibly most important, it would provide a means to understand the lung as a lung, and not as a collection of type II cells, or regional areas of poor air exchange, or thrombosed arteries, or whatever the glaucomatous monodisciplinary approach focuses upon.

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Fourth, we would understand the lung as a system of total pathology. Our one team of investigators, working with one animal model, will build up within its own members various centers of specialization: physiological, histopathological, and biochemical. These functions complement and mirror the similar tripartite functions of a clinical pathologist in his daily work-- and it is a clinical pathologist who who is senior investigator. So another significant product is the entry of clinical pathology into the fundamental research on lung disease. By professional necessity, this specialist is accustomed to thinking of the patient in broad terms, inclusive of formal histopathology but also of the biochemistry of body fluids and the physiological function of organs and systems.

Indeed, the molecular fractionation of homogenized lung, and the serial examination of such fractions for their relevant enzymatic, macromolecular, and neurohumoral components is very much along the line of multiphasic screening. The major difference is, we have chosen to measure moities that we suspect in advance of having important cellular roles for lung function, growth, injury, and repair.

Ordinarily, to achieve these magnitudes of significance would be impossible on the proposed operational funding basis within the specified time. In this case, NIHL has generously funded a team which is laying the groundwork for every area outlined in the proposal above except those in which The Council for Tobacco Research, through previous support, has already provided us with facilities and expertise. The only new endeavor is with reference to neurosecretory granules.

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f. Summary:

The overall significance, we hope, will be to turn lung researchers and clinicians away from monodisciplinary studies towards multidisciplinary ones; away from consideration of the lung as a breathing bag and towards consideration of it as a tissue with all the homeostatic, regulatory, and macromolecular functions an organ requires to maintain its own autonomous existence; away from passive models of lung function towards those in which muscle contraction, tissue metabolism, and neurohumoral secretion are intrinsic to functional activity; away from emphasis on the alveolar transfer function of the lung and towards emphasis of bronchiolar function, compliance, and gas-mixing properties; away from a philosophy of mass lung action and towards one in which unit lobules dominate lung activity in the same way that unit nephrons dominate renal activity.

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PROJECTED SCHEDULE OF LABORATORY WORK

PHASE	MONTH	DUTIES FOR PERSONNEL
START-UP	1	Advertise positions; interview and hire candidates. Order equipment. Begin technical training. Breed guinea pigs.
TRIALS & DEVELOPMENT	2-3	Dry runs on molecular biology. Practice runs on autopsy/histology. Develop neurosecretory granule fluorescence technic.
	4-8	Practice runs on total experiment.
	9	Feedback modulation of experimental design.
EXPERIMENTS	10-14	First series of experiments: cycle 1 <u>oxygen toxicity</u> , 20 guinea pigs, fixed starting age.
	15	Finish enzyme work on frozen extracts of gradient fractions.
	16	Calculation of results. Write paper. Feedback modulation of experimental design.
	17-23	Repeat cycle 1
	24-30	Second series of experiments: cycle 2 <u>oxygen toxicity</u> , 20 guinea pigs, various starting ages (effects of growth on injury and repair)
	30-36	Third series of experiments: cycle 3 <u>Tobacco induced</u> alterations, 20 guinea pigs (correct model to be chosen from cycle 1 or 2 above) Final report.

FULL-TIME DUTIES OF PERSONNEL

Biologist:	Does tests for and evaluates clinical data. Breeds animals, keeps records, assists in autopsies and histopathology. Does mucus cell counts and tantalum macrophage counts, assists physiologist, prepares fluorescence specimens for pathologist. Serves as group leader.
Physiologist:	Runs blood gas station, does multichannel physiological testing, maintains equipment, evaluates data, performs computations, assists biologist and/or chemist.
Chemist:	Performs gradient fractionations, enzymatic analyses, ribosomal analyses, thin layer chromatograms, evaluates data.

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(5) INTERPRETATION OF RESULTS

This will be considered under 4 subheadings: molecular, morphological, clinical, and theoretical. In reality, the experiments are performed as a whole, and there are not separate experiments in each category. Animal models will be examined at 4 days, 2 weeks, and 4 months, which are respectively interpreted as acute, subacute, and chronic.

a. Molecular:

1. Ribosomal activity and protein synthesis:

It is assumed that the three proteins considered, the enzymes glutamyl transpeptidase, alkaline phosphatase, and aspartyl transcarbamylase, are produced by ribosomes in the classic manner.

- (a) Glutamyl transpeptidase: studies on liver (58) or isoenzymes (59) suggest strongly that this cytoplasmic enzyme appears in the serum during the early phases of cellular damage. Thus, we shall try to correlate quantitatively levels of this enzyme in homogenized fractionated lung with levels of it in the serum during injury and repair. In addition, we hope to pinpoint the locus of intracellular synthesis pari passu, since the fractionating procedure deals simultaneously with enzymes, subcellular particles (such as vesicles derived from rough endoplasmic reticulum), polysomes, and ribosomes. This remarkable capability is inherent in the process of zonal ultracentrifugation. Hence, the interpretation of results deals with molecular events in the cell in terms of their morphological consequences, and holds possible advances both in clinical and theoretical knowledge.

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- (b) Aspartyl transcarbamylase: this enzyme is nucleus-related, for it is an obligate part of the DNA synthesis chain.

Whereas DNA may also be studied directly (by thymidine labeling) with the indirect aspartic transcarbamylase technic it may prove possible to infer, from serum changes, events which occur in an organ. Thus, enzyme levels in serum will therefore also be correlated with their corresponding tissue values and their location during fractionation procedures, as above.

- (c) Alkaline phosphatase: just as glutamyl transpeptidase is a marker for injury, and aspartic transcarbamylase for cellular regeneration, alkaline phosphatase is a marker well known for its association with differentiated mucosal cell activity. Results of all three enzymes will be interpreted in this light. In addition, because the alkaline phosphatase enzyme system is so well known, there are ready methods for its histochemical and serum isoenzymatic analyses, both of which we propose to do. Thus, the differentiation of cells containing alkaline phosphatase will be studied from the histochemical and as well the subcellular aspects, and also with respect to isoenzyme where that seems feasible. Since the differentiated function of the bronchiole includes, we believe, airways regulation, these data will also be correlated with lung function studies.

- (d) Ribosomal protein: We have conclusively showed the regular appearance of abnormal constitutive ribosomal protein early in lung injury with oxygen. We shall continue to use it as

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a marker in this way. It will be of great interest to correlate changes in enzymatic spectrum with changes in ribosomal structure. If we are lucky, we may get some clues to the molecular nature of the abnormally dense contents of rough endoplasmic reticulum (of Clara cells) which appear during the first week of oxygen intoxication (7,20) and probably other lung injury.

2. Neurosecretory granules: whereas proteins and ribosomes are intricately related, the subcellular site of Kultschitsky cell active secretion is extra-ribosomal and is unknown. Our first interpretations, then, will be addressed towards the subcellular localization of these granules in molecular dissections on the ultracentrifuge and correlated by light and electron microscopy; and second, we shall interpret regenerative and synthetic activity of the various hydroxyindole family members with the state of lung injury and repair. These interpretations will be colored by our notion that the neurosecretions may play a regulatory role in the maintenance of lung lobular homeostasis and/or function, themselves measured by lung function as well as molecular enzyme studies.

b. Morphological

1. Fluorescence microscopy: this is the qualitative tissue method for citing the localization and distribution of neurosecretory granules isolated on the ultracentrifuge.
2. Electron microscopy: this is a consultative service, the function of which is to provide the morphological correlation for the molecular processes measured above. Pictures will be interpreted qualitatively, with special regard to mucosal cell patterns, and to distribution of neurosecretory granules.

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3. Light microscopy:

- (a) Tantalum in macrophages: this is interpreted as a means of roughly gauging lung clearance, and based on quantitative counts.
- (b) Goblet cell counts: these are interpreted as indicative of the animal equivalent of human mucus gland counts. Chronically increased numbers of goblet cells are interpreted as indicative of chronic bronchitis.
- (c) Semiquantitative graded lesions: these form the basis for considering whether our animals have in fact developed comparable lesions. We assume, in the interpretation, that there are some true parameters and some independent variables. This ordinal grading is a parameter. Only animals with similar toxic dose histories and similar grading will be compared. (Past experience has been that correlation coefficients of the order of 0.9 are possible if graded bronchiolar morphology is compared to oxygen dose.)

c. Physiological ("clinical") lung tests:

- 1. Nitrogen washout procedures: These are interpreted with respect to regional disorders, the numerical lesions above, and molecular bronchiolar changes. The oxygen washout measured by the high-speed analyzer is the inverse of the nitrogen washout, as discussed above, and nitrogen washout is a time-honored method for the determination of regional \dot{V}/Q anomalies. This is a particularly reasonable approach in the guinea pig lung, because the collateral air circulation (pores of Kohn) is poorly developed in these animals (60). Thus, the guinea pig should emphasize, by nature's design, factors that lead to lobular airways obstruction.

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2. Minute ventilation: integration of the total tidal exchange per minute, as measured by a body plethysmograph, provides this datum.

Incidental to this, the breathing frequency is also provided. These data are cross-correlated with nitrogen washout primarily, and with molecular and histocytological data secondarily.

d. Chest roentgenographs: Insofar as feasible, these are interpreted as the in vivo counterparts to histological examination and particularly subgross anatomy, at the bronchiolar level, and correlated with these.

e. Complete autopsy procedures provide a measure of comparability of the accumulated clinical data obtained during one life, and both sets of information are useful in establishing the comparability of measurements in serried experiments. This is quite important, because the experimental design calls for the continuance sequencing of pairs of guinea pigs rather than mass experiments on a large number of guinea pigs at once.

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(6) PREVIOUS WORK

Previous advances made possible in our laboratory by dunded support, exclusively by The Council for Tobacco Research, have been molecular, morphological, and theoretical. However, before the current proposal, we had no means of physiologically testing any practical conclusions made as a result of our studies.

a. Molecular:

We have demonstrated that, contrary to expectations based on early histochemical work (Sorokin), there is a sharp drop in ribosomal synthesis in lungs of normal newborn guinea pigs a few days after birth (50). These observations themselves devolved from the creation of a whole technology in the molecular dissection of lungs, one in which our laboratory seems to be foremost at present (3 ,50,52, 53,61,62,63). With this technic, we were able to demonstrate that ribosomal synthesis is turned on by mucosa soon after the destructive lesions of pulmonary oxygen intoxication. Moreover, we showed that ribosomes of injured lungs differ molecularly from normal ones through electrophoretic differences in the constitutive basic protein of the ribosome, a finding of fundamental importance throughout the field of cell biology. Experiments to localize the findings to ribosomal subunits have not as yet been decisive. Lately, we have correlated distribution of the isoenzymes of alkaline phosphatase with these ribosomes in growing and injured lung (4). Results thus far show rather pronounced shifts in the subcellular distribution of alkaline phosphatase, absolutely as well as relatively, some of which seem related to ribosomes. Three different species of rodent -- rabbit, rat, guinea pig -- share some similarities. Most intriguing is the

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observation that the mass of an individual class subcellular alkaline phosphatase particle generally increases with age, and also that some particle sizes seem to have characteristic locations in terms of Svedberg ultracentrifugation. Figs. 15-20.

b. Morphological:

The bronchiolar injuries of oxygen have been clearly documented in rat (20), mouse (49), guinea pig (7), and human (21). This is a unique contribution of our group, since all other investigators of oxygen intoxication have concentrated upon alveoli instead of bronchioles. Our studies have included ultrastructure also (20,64). We have showed the progression of the oxygen-induced lesions, and in particular, have documented the important role played by bronchiolar reaction and metaplasia in airways obstruction (20), a role for which exfoliative cytology provides a handy clinical foil (65). We developed the first concept of oxygen "dose". Under the impetus of these studies, we proposed a semiquantitative ordinal grading system (Fig. 14) for evaluation of lung morphology which helps eliminate observer bias in the comparison of histological lung findings. This system is a direct outgrowth of a similar one used in the evaluation of bronchopulmonary dysplasia (oxygen intoxication of the newborn), the study which led to our Council for Tobacco Research support (21). In addition, we have completed the first survey by any group of the developmental and ultrastructural morphology of human bronchioles (11). Currently, we are summarizing a portion of this work in each of three different review volumes (13,14,15). Figs. 21-26

c. Clinical:

The clinical dangers of oxygen for the lungs of newborns were first explained by us (66). Subsequently, we coined the term "bronchopulmonary dysplasia" as

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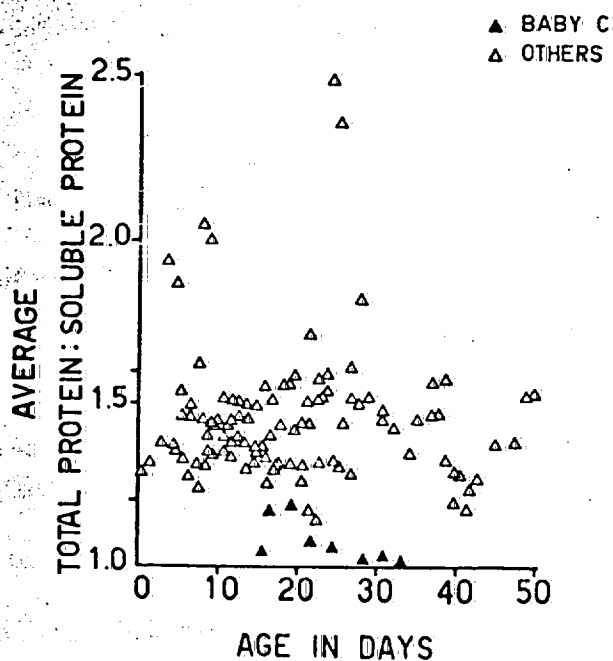
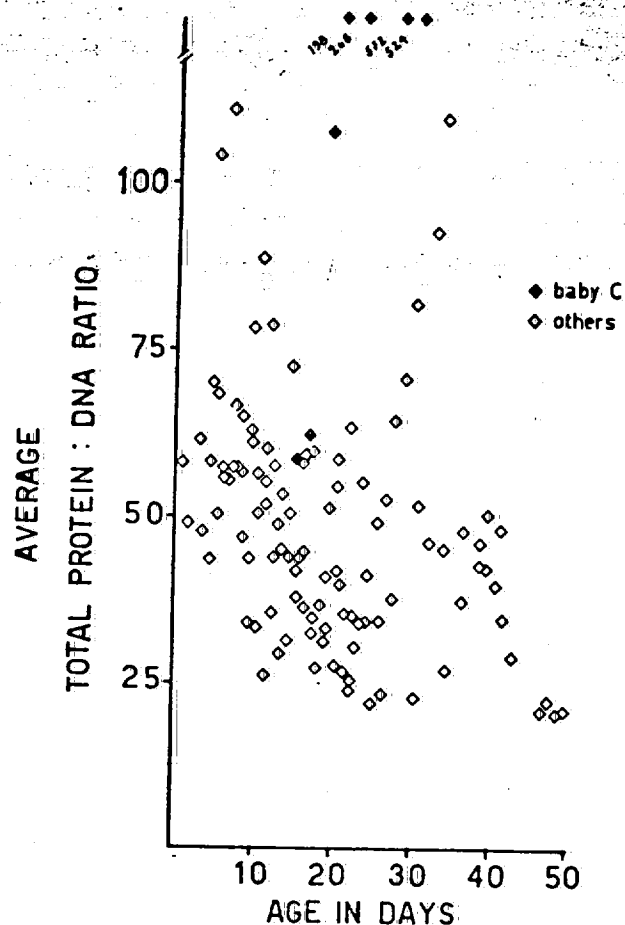
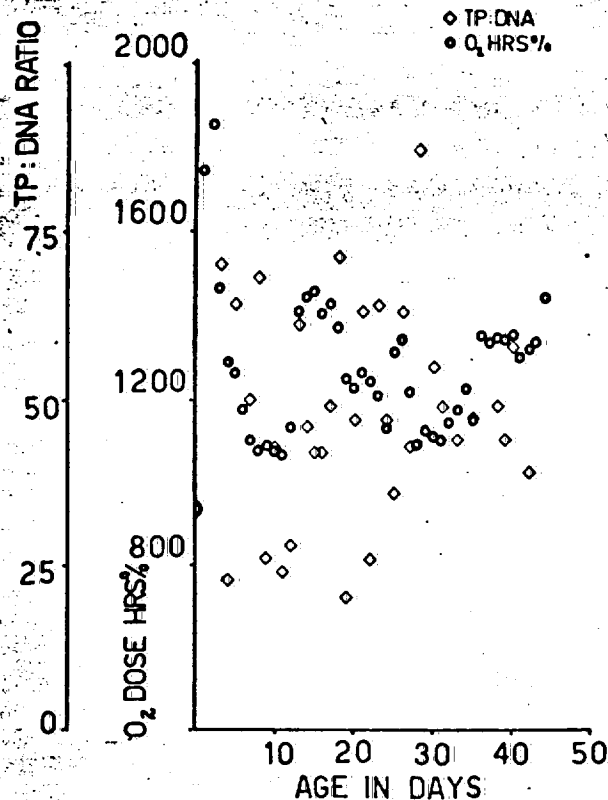


Fig. 21 COMPARISON OF BIOCHEMICAL VARIATIONS IN 24 HR POOLS OF ENDOTRACHEAL SECRETIONS IN 5 BABIES WHO DIED AND ONE WHO SURVIVED AFTER OXYGEN THERAPY.

Upper left: Baby C has least oxygen dose

Upper right: Baby C secretes much more protein than DNA. This tends to show that oxygen necrotizes cells.

Lower right: Baby C secretes much more soluble than insoluble protein; the latter is associated with increased production of mucus and fibrin.

Conclusion: There may be an association between the biochemical and clinical effects of oxygen toxicity.

Added note: Cytological analyses of the above secretions showed many mucosal cells with atypia present (as well as lining cells from other lung regions.)

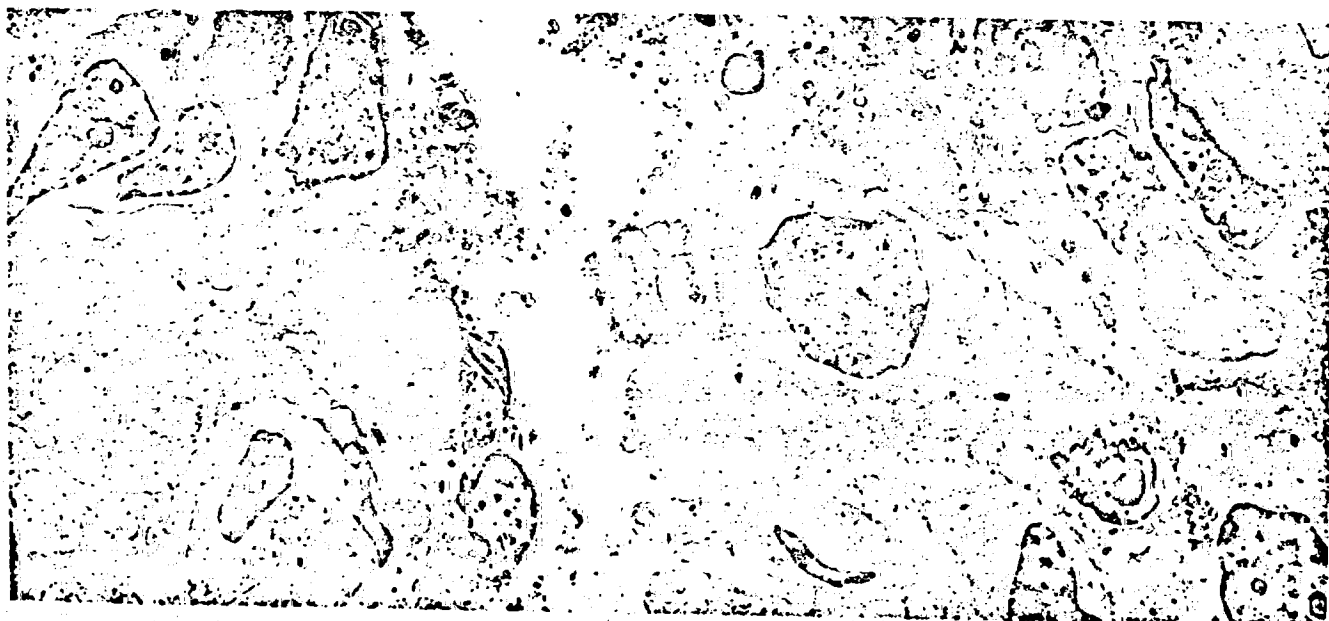
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FIGS. 22, 23, and 24: With collaboration of J M Lauweryns.

OXYGEN INTOXICATION OF THE END-BRONCHIOLE OF THE
NEONATAL GUINEA PIG (Composite photograph)

This picture develops within 4 days of the start of 100% oxygen therapy at controlled humidity. We believe that the extensive damage to the "valve" area of the bronchiole is much like that seen in humans. In Fig. 22, which is a montage of several photographs, the swollen smooth muscle on the extreme sides of the picture is of note, as well as the necrotic mucosal, Clara, and ciliated cells. In Figs. 23 and 24, the damaged Clara cell is seen, and the characteristic rough endoplasmic reticulum with its dense fibrillogranular contents is apparent. Mitochondria are known to be easily injured by oxygen; and the close association of (?regenerative?) ribosomal organelles with injured mitochondria is often seen in these experiments.

Fig. 22

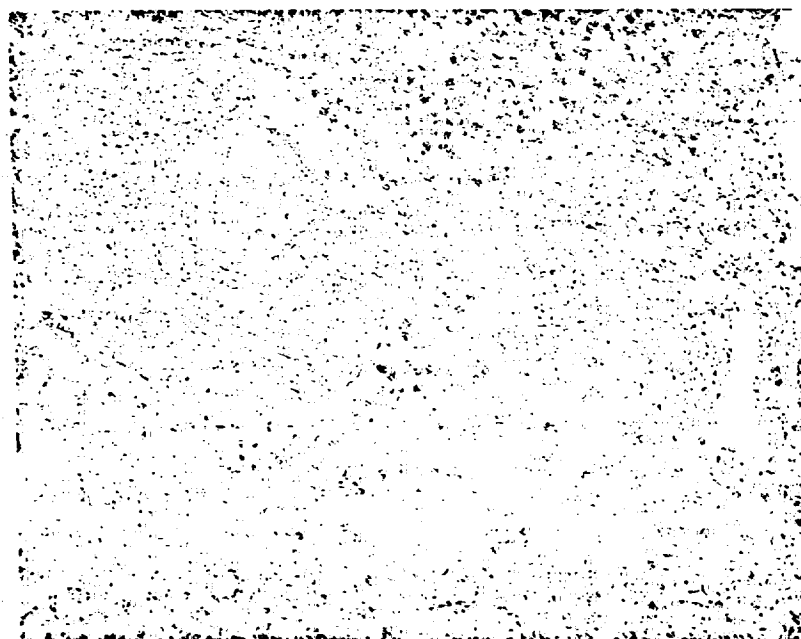


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Fig. 23



Fig. 24



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descriptive of this rather characteristic condition (21) and the term is slowly coming into usage. We have documented that airways obstruction may begin in newborns and leave chronic residual disease (67). We have showed how roentgenological details may be followed with regard to prognosis (68), and the same for exfoliative cytology (65). The molecular pathology of the secretions has been explained (28). Even in our first full report, we had developed an ordinal grading method for interlaboratory comparison (21). Along the way, we have tested and rejected the notion that hyaline membrane disease is a sequel to morphological brain injury, and developed the first systematic neuropathology for long-term survivors of that condition (29). More recently, we worked out the difference in morphology between long survivors who do and who do not have a history consistent with oxygen intoxication. It turns out that hyaline membrane disease alone may heal by a type of chronic interstitial pneumonitis, whereas bronchopulmonary dysplasia is associated with chronic obstructive airways disease, chronic pulmonary hypertension, and chronic metaplastic lesions of the bronchioles (21,69). In view of the importance of juvenile chronic bronchitis to adult obstructive lung disease, we think these are significant observations. Figs. 21,25,26,28

d. Theoretical:

One major contribution has been the idea of the unit lobule (10,14), a rather unique solution to many of the problems which arise in the interpretation of neonatal lung disease and its sequelae. Briefly, this theory lays heavy stress on the importance of the bronchiolar components of the ontogenetic lobule for regulation of air, lymph, and blood flow (10,11,15), and also the intersaccular septa (which are the unique infantile lung structures described by Professor J. M. Lauweryns) for the modulation of mechanical and homeostatic behavior.

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The theory emphasizes consideration of the lung as tissue. The emphasis on bronchiolar compliance has recently been supported by in vivo measurements of infants in our clinic, which have shown compliance of so great a magnitude that carbon dioxide retention and ventilatory ("dead-space") acidosis are regular concomitants of some types of positive end-expiratory ventilation. The last is itself a clinically significant observation. It is interesting that our theory feelings converge with those recently expressed by J. M. West: ".....a cynic might say that only a respiratory physiologist with his obsession for simple diagrams could picture an alveolus as a balloon on a tube exposed to pleural pressure." (60). That "tube" and the regulation of its compliance will be, we believe, among the principal areas of advance in the next few years of lung research. We specifically postulate that injuries to lung during the neonatal period of rapid growth may lead, in some cases, to life-long pulmonary handicaps and a predisposition to chronic airways disease. Figs. 27, 28.

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Fig. 25

Saccular portion of the normal lobule of the prematurely born infant (c. 600 g)

Especially noteworthy are the thick "intersaccular septa", the well-developed and almost mature air-blood barrier portion of the capillary-epithelial junction, the numerous glycogenolated "cuboidal" cells, and the neurosecretory cell which is far from any observable mucosa (circle).

By collaboration with J M Lauweryns



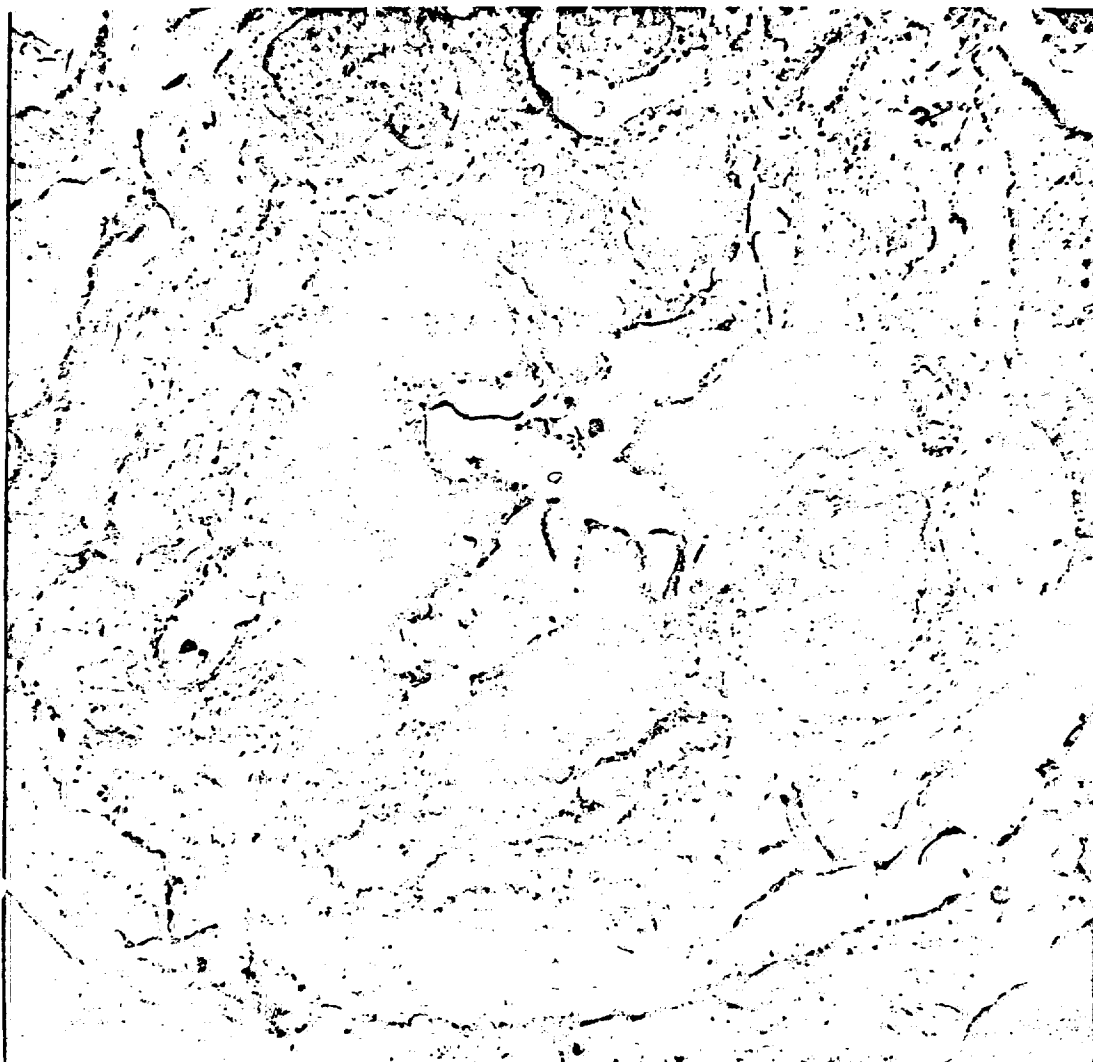
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Fig. 26

Destruction of the saccular portion of the neonatal lobule after more than 250 hr of 100% oxygen with assisted ventilation.

By collaboration with Gladys Harrison.

Especially noteworthy is the disappearance of air-blood barriers, cellular atypia, and prominent interstitial collagenization.



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FIG. 27

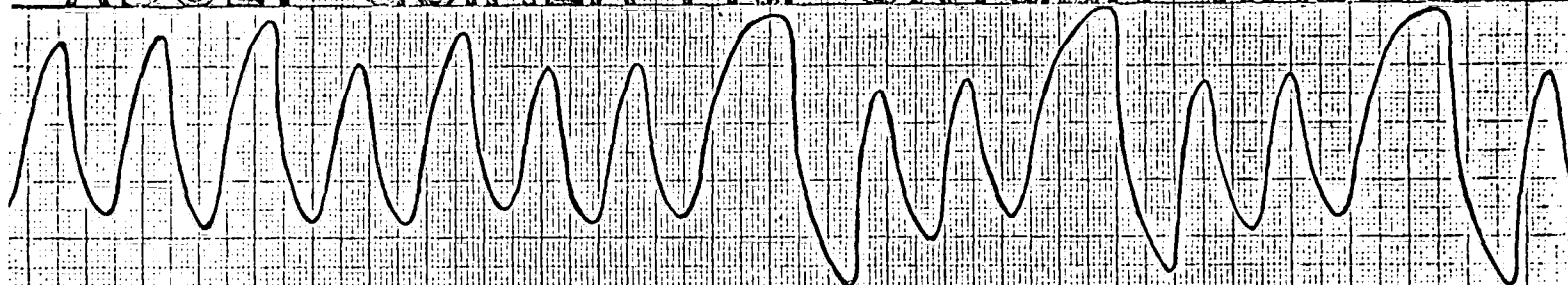
Breath-by-breath analysis (retracing of actual record) of oxygen extraction from normal room air for an adult guinea pig. The scimitar-shaped tracings are an artefact of the (non-rectilinear) Harvard Apparatus Company's pen-amplifiers, and would be replaced in the current proposal.

The record shows the practicality of making fast oxygen measurements in the plethysmograph (see Fig. 8) used in this project. The shape of the decay curve, after 5 minutes of breathing 100% oxygen, can be measured easily as these data--which were secured after breathing room air--show.

If the oxygen extraction by a guinea pig lung can be measured at his nose, it should be practical to do the same for patients. Thus, it might be possible to envision a non-invasive continuous monitoring system for patients taking physiological function tests, or for those who must receive mechanical ventilation.

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ADULT GUINEA PIG OXYGEN WASHOUT



SCALE:

DATE: SEPT. 28, 1972

1 SECOND

WEIGHT: 350 GRAMS

1.0% ΔO_2

LOCATION OF SAMPLE
TUBE: AT NOSTRIL

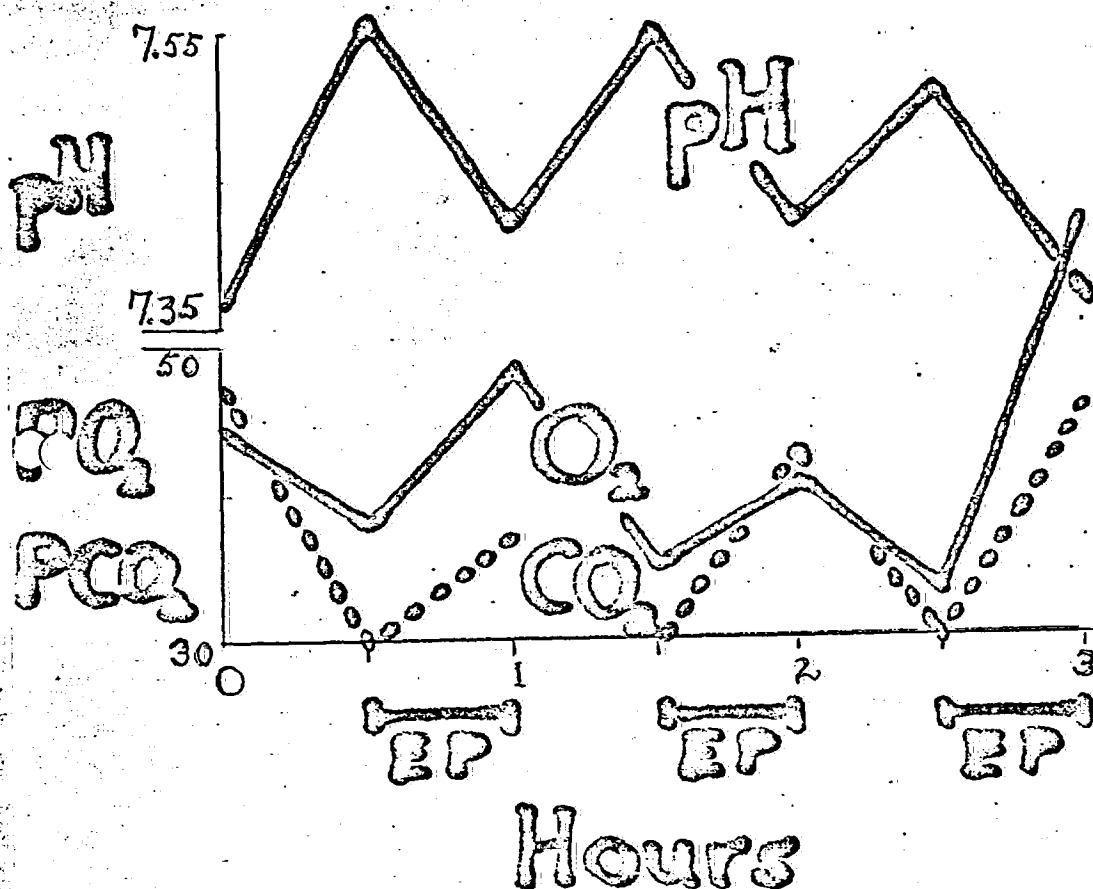
MOUTHPIECE: NONE

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FIG. 28

Effect of positive end-expiratory pressure on bronchiolar compliance

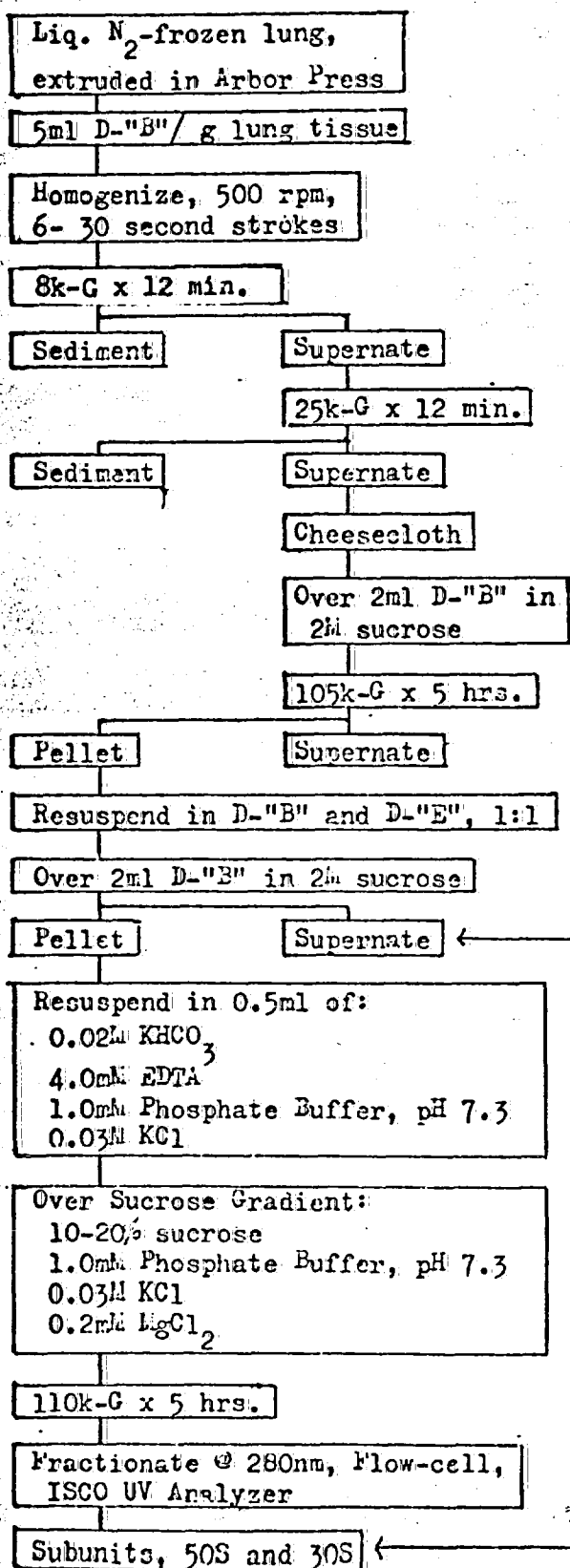
This figure shows the importance of an appropriate lung model to clinical medicine. The results below should not occur in a surfactant theory.



The application of end-expiratory pressure continuously for half hour periods (about 6 mm H_2O pressure) helps drive oxygen into the lung, for the P_{aO_2} increases. However, the pressure also increases P_{aCO_2} by virtue of increasing the bronchiolar "dead-space". The net effect may actually worsen the acidosis as above. However, this latter is unusual. What the experience demonstrates is that the neonatal bronchiole is highly compliant. In this the case, the patient weighed about 1200 g.

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Flow Diagram for Isolation of Ribosomal Proteins from
Subunits Derived from Newborn Human Lungs



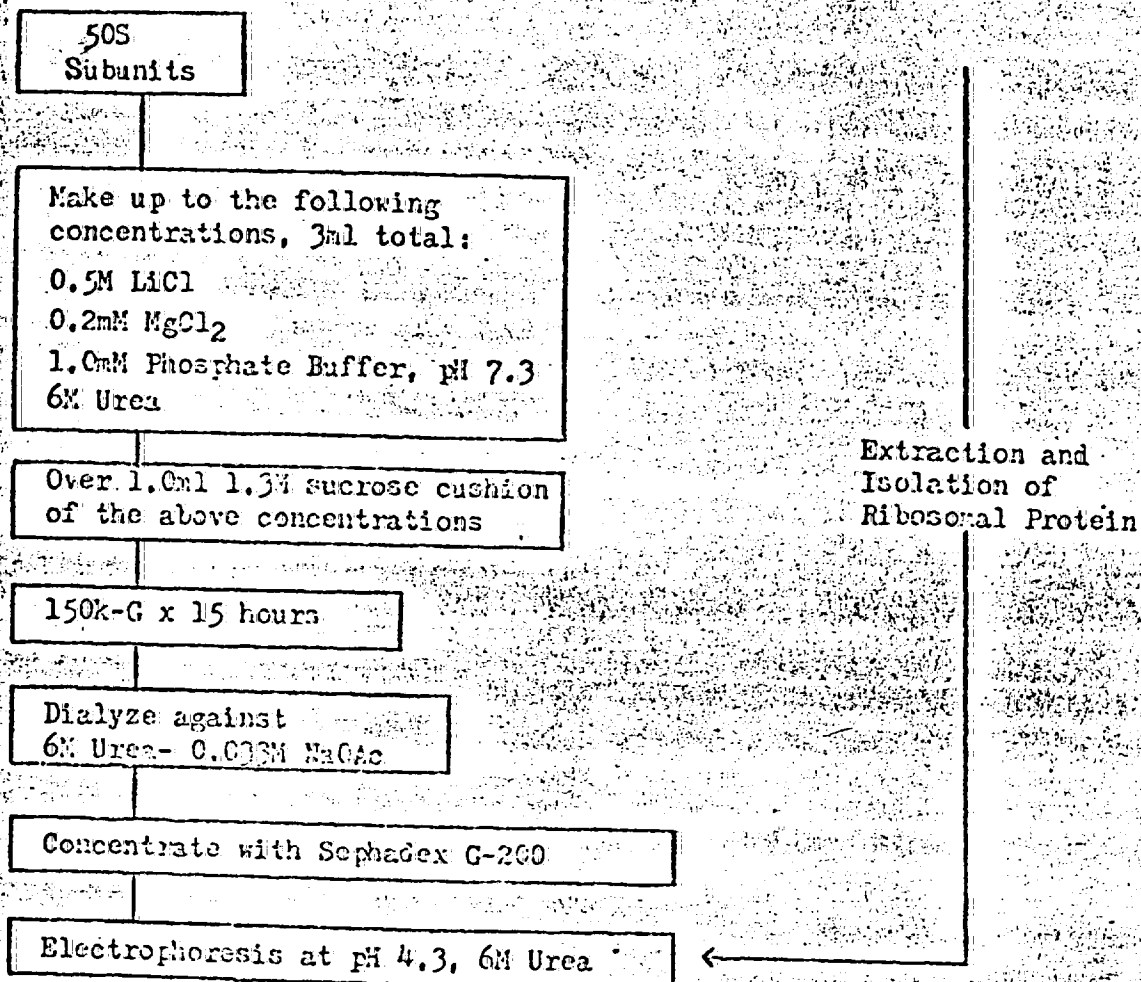
Preparation of Monosomes

Preparation and
Separation of
Ribosomal Subunits

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77

Flow Diagram, continued



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1003538708

901 WEINBAUM

1003538709

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 2, 1973

Grant Application No. 901

To: The committee comprising Drs. Gardner, Loosli and Meier
Subject: George Weinbaum, Ph.D., Albert Einstein Medical Center,
Philadelphia, Pa.
New application No. 901
"The Effect of Air-Bourne Pollutants on Lung Proteinase:
Antiproteinase Levels"

History

This proposal was Case No. 147 and full application was suggested.

The request is for \$45,332 plus two years.

Documents Submitted.

Attached is an application dated 1/15/73.

(Reprints of recent papers listed on page 16 were provided; they will be forwarded if so requested).

Comment

At our request, Dr. Weinbaum is providing detailed information on techniques for smoke exposure of animals; this will be forwarded when received.

FWM
F.W.N.

FWN:wg
Encl.

1003538710

Dr. Gardner
Dr. Loosli
Dr. Meier

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 50TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant

JAN 31 1973

Date: 1/15/73

(Use extra pages as needed)

Co-

1. Principal Investigator (give title and degrees):

GEORGE WEINBAUM, PH.D., Bioscientist, Pulmonary Disease Section
PHILIP KIMBEL, M.D., Head, Pulmonary Disease Section

2. Institution & address:

Albert Einstein Medical Center
Northern Division
York & Tabor Roads
Philadelphia, Pa. 19141

3. Department(s) where research will be done or collaboration provided:

Pulmonary Disease Section and
Research Laboratories
Albert Einstein Medical Center

4. Short title of study:

THE EFFECT OF AIR-BOURNE POLLUTANTS ON LUNG PROTEINASE:
ANTIPROTEINASE LEVELS

5. Proposed starting date: July 1, 1973.

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6. Estimated time to complete: 3 years.

7. Brief description of specific research aims:

The twin objectives of this investigation are 1) to identify and quantitate possible factors responsible for protecting the lung against the action of autogenous proteinases - previously shown in this laboratory to produce experimental emphysema in dogs - and 2) to examine the role of cigarette smoke and other pollutants on this interaction. We shall isolate, quantitate and characterize the normally occurring substances, found in lung tissue and sera of several species of animals, which inhibit the activity of proteolytic enzymes. These data will then be compared both with the incidence of spontaneous emphysema and with the ease of experimental emphysema induction in these species. The production of antiproteinases and their ability to interact with enzymes able to induce experimental emphysema in animals will be studied and evaluated in normal animals and those exposed to cigarette smoke or nitrogen dioxide. Both of these components are known air pollutants and the latter agent has been shown to cause emphysematous lesions in animal models.

Using experimental pollutant and nonpollutant conditions we will look for qualitative and quantitative changes in levels or activities of autogenous proteinases and anti-proteinases and determine the relative effectiveness of emphysema induction in the dog model system using leukocyte or macrophage proteinases in both conditions.

8. BRIEF STATEMENT OF WORKING HYPOTHESIS:

Our working hypothesis is that emphysema may be induced by the proteolytic activity of specific enzymes from polymorphonuclear leukocytes or pulmonary macrophages. The release of these enzymes may be stimulated by various air-borne pollutants. These enzymes overwhelm the local defense mechanisms in the lung, including such factors as serum and tissue antiproteinases, and destroy or alter the elastic tissue of the alveoli.

9. DETAILS OF EXPERIMENTAL DESIGN AND PROCEDURES

PART I - INTRODUCTORY STATEMENT

Epidemiologic studies suggest that smoking is an important factor in the development of pulmonary emphysema in humans. Two major, non-mutually exclusive theories for its induction differ only in their emphases on initial tissue attack. In the vascular theory (1), the initiating lesion is of vascular origin; resulting in obstruction of branches of the bronchial or pulmonary blood supply. The subsequent deficiency of nutrients would then lead to necrosis of alveolar walls and septa to form the characteristic emphysema pathology. According to the second theory (2), emphysema develops due to the direct attack of proteolytic enzymes at the air-lung interface. Regardless of which theory is correct there is eventual extensive damage to both the vascular and parenchymal tissue. A logical source of this degradation could conceivably be intracellular collagenase and/or elastase, released into the surrounding tissues due to cellular necrosis caused by such stimuli as chronic infection or chronic pollution-induced destruction.

Although cigarette smoking and human emphysema have been statistically related using clinical and autopsy material, it has not yet been shown if there is a direct causal relationship or if smoking and other pollutants merely accelerate a normal aging process. Also, if there is a direct relationship between them it is still possible that smoking is but one of a number of extrinsic factors able to act synergistically with autogenous agents to effect this lung damage. Those experimental studies which have been performed on the effects of smoking do, however, suggest reasons for exploring the action of cigarette smoke as a single contributing factor in emphysema induction.

The response of the lungs to nitrogen dioxide, especially to chronic low levels, should similarly be investigated using the same criteria used for smoking. Not only has this very simple compound been shown to produce emphysema (3), its occurrence in cigarette smoke and other complex forms of pollution suggest its use as a model causative compound of simple structure.

When human pulmonary leukocytes from smokers and non-smokers were compared (4, 5, 6), even asymptomatic smokers had greatly increased numbers of primarily polymorphonuclears and macrophages with a direct cor-

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relation to the amount of smoking and smoke inhaled. That this is not simply a protective effect, i.e., more phagocytes are supplied to remove an increased amount of foreign matter, was demonstrated by the finding that there was an actual decrease in the antibacterial activity of alveolar macrophages from smokers. This effect differed among the various brands tested and varied inversely with the effectiveness of filtration (7). The significance of this particular change in capability, that is, the decrease in antibacterial activity as opposed to such other findings as change in size or pigmentation, lies in the ability of the phagocytosed particles to cause the lysis of the engulfing leukocyte with subsequent release of enzymes able to degrade pulmonary tissue.

The demonstrated greater susceptibility of cigarette smokers to respiratory infections may, therefore, be due to this combination of insufficient leukocyte degradation of invading organisms and the damage resulting from the liberated proteases on lung tissue (8). Since studies of human pulmonary emphysema are hampered by time required for induction and moral considerations, animal models have been employed. Although the horse has the advantage of having lung anatomy, distribution of bronchial arteries, and a natural emphysema similar to that found in humans, economy dictated that another animal model be used. Since this laboratory has successfully produced emphysema-like lesions in dogs (9), using aerosolized leukocyte proteinases and since the parenchymal effects of smoking in dogs resembled human emphysema (10) this system will be used.

It has been suggested by many groups (11) that a decreased level of serum alpha₁ antitrypsin is of primary importance in correlating with the development of hereditary emphysema. Other investigators (8,12), however, have been unable to demonstrate a relationship between the development of emphysema and smoking in those having intermediate or normal levels of this antiprotease. Although this may be a characteristic of the species studied, the report (13) that lung tissue binds the serum antiprotease suggests that not only is the total level of antiprotease important but also its localization and its availability to bind and thus inhibit proteolytic enzymes. Material from fractionated lung tissue must be examined for its role in proteinase-antiproteinase balance.

With these data in mind, we propose the following research program.

PART II

A. RESEARCH PLAN OUTLINE

1. To Characterize Antiproteinase(s) in the Lung.

a. Fractionate lung tissue from several mammalian species and isolate and characterize the factor(s) responsible for antiproteinase activity.

b. Comparison of the factor(s) found in the lung with serum proteins demonstrating antiproteinase activity.

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2. To Determine the Role of Smoking and Other Pollutants on the Interaction between the Proteinases and Antiproteinases in the Lung.

a. In vitro assay of proteinases from alveolar macrophages, lung tissue; and PMN's and the qualitative and quantitative effects on them due to exposure to cigarette smoke or nitrogen dioxide.

b. In vitro assay of serum and lung antiproteinases from normal and pollutant-treated dogs.

c. Comparison of ease of induction of experimental emphysema using material from normal and pollutant-treated dogs on both in vivo and in vitro levels.

B. EXPERIMENTAL PROCEDURE

1. Characterization of Lung Antiproteinases

a. Species variations and relationship to spontaneous emphysema.

Lung tissue from normal guinea pigs, hamsters, humans, horses and dogs will be examined for substance(s) able to inhibit any of the proteolytic enzymes found in polymorphonuclear leukocytes. These particular species were chosen for the following reasons. In a study by Ihrig, et al (14), guinea pigs had the highest and hamsters the lowest levels of serum antitryptic activity of those animals tested. Since preliminary results in this laboratory (15) indicate differences in the ability of these animals to develop emphysema following exposure to papain we believe a better characterization of the various lung protease inhibitors is warranted. Human material will be studied not only as this is the species of primary concern, but also because no clearcut relationship has as yet been found between the intermediate levels of serum proteinase inhibitor and development of emphysema in smokers (12). One approach, that of genetic analysis of the numerous alleles involved in production of serum alpha₁ antitrypsin is being undertaken in several other laboratories (13). Our approach is a biochemical one in which we will evaluate the role of proteinase:antiproteinase balance under pollutant and non-pollutant conditions in relation to the development of experimental emphysema. Horse tissue will be examined as this species is one of the only two (along with the rabbit), other than man, recorded as developing spontaneous emphysema. Additional considerations suggesting that the horse is a useful model are (a) its level of serum inhibitor is approximately the same as the level in humans; (b) lung anatomy in the horse more nearly resembles that in man; (c) the time course of emphysema development, adjusted to human years, is quite similar between the two species. Dog material will be employed due to the considerable body of data already available from this lab using this animal. Also, a direct relationship was found between macroscopic parenchymal disruption in dogs and duration of daily cigarette smoking over period of 0-22 months (16). Microscopically, these lesions resembled human emphysema (16). Emphysema has been successfully produced in our laboratory in vivo

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using papain or leukocyte homogenate instillation or aerosolization. We have also developed an in vitro system for emphysema production utilizing isolated lobes and instillation of proteinases.

Isolation and Quantitation of Tissue Inhibitors

Lung tissue from the various species will be minced, homogenized in a blender, and washed three times with water followed by centrifugation at 15000 rpm (15). The pellet will be washed twice, including once overnight, with 1 M NaCl. The sodium chloride supernate will then be recentrifuged at 42,000 rpm (15). Supernate extracts will be assayed for their ability to inhibit the proteolytic activity of enzymes extracted from polymorphonuclear leukocytes using the hemoglobin assay (17). It is expected that antiproteinase activity will be found in this fraction because of the preliminary work of Lieberman (18) and Janoff (19). We will initiate this work using the NaCl extract, but will check other lung extraction procedures also.

Characterization of Inhibitors

In order to characterize these antiproteinases they will be purified using such standard procedures as precipitation with ammonium sulfate, methanol, trichloroacetic or perchloric acid and various types of ion-exchange chromatography (20). An alternative procedure will be sought using the principle of affinity chromatography (21). Crystallin trypsin will be bound to Sepharose 4B using the cyanogen bromide method of Cuatrecasas and Anfinsen (22), and the matrix poured into a column. Lung extract containing the antiproteinase will then be applied to the column, nonbound protein washed off, and the adhering inhibitor selectively eluted.

Effect of Inhibitors on Induction of In Vitro Emphysema

In vitro emphysema has been induced in our laboratory using isolated lobes of dog lungs. Following removal of the lungs from the body the individual lobes were instilled with solutions of proteinase(s) and the enzyme(s) allowed to digest the tissues for 1½ hours at room temperature. We will determine if the inhibitors isolated above will affect in vitro induction. Formaldehyde will be instilled into the lungs at a standard pressure of 25 cm. The organs will be immersed in formaldehyde for 48 hours, mounted, sliced and stained. Sections will be examined for alveolar wall destruction using the method of Dunhill (23). This procedure will allow us to better define what we are considering to be emphysema-like lesions and also to quantitate the effects of various doses of agents required to produce a defined level of lung destruction. The use of isolated lobes will provide us with a considerable economic advantage over in vivo work employing the entire animal. It will also allow us to minimize the effect of any animal to animal variation on a given experiment.

b. The Relationship between lung and serum proteolytic inhibitors

Although both the serum and the pulmonary tissues have been reported to contain substances able to inhibit proteolytic enzymes no one has studied the possible relationship between the inhibitors from

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these two sources. The value of such a comparison lies in the relationship of these inhibitors to both the etiology and prognosis of the disease state, always with the assumption that since proteolytic enzymes are significant in these processes so too are these inhibitors. If a serum inhibitor is actually a subunit of a lung inhibitor (or vice versa) then it could be expected that there would be some sort of quantitative relationship between their concentrations. Conditions regulating this ratio could be involved in pathogenesis of pulmonary emphysema. Genetic abnormalities affecting the specific regulatory gene(s) would also be expected to affect both serum and pulmonary inhibitor if they were derived from the same molecule. Conversely, if the lung and serum antiproteases are not subunits of some primal molecule any relationship between the concentration of one vs. the other could have some non-genetic, e.g., environmental origin.

Several methods will be employed to study the relationship between antiproteases of the lung and the serum. Lung antiproteases isolated as described in specific aim "a" will be examined using polyacrylamide gel electrophoresis, with and without sodium dodecyl sulfate, to determine if these agents isolated from different species have subunits such as easily dissociable polypeptide chains and if they are similar to serum antiproteases isolated in a similar chromatography procedure. Antisera will be raised in rabbits injected with purified serum antiprotease and tested against lung antiprotease using the immunoelectrophoretic and Ouchterlony techniques to find out whether or not the molecules share antigenic, and therefore, structural sites. Samples of each inhibitor will also be hydrolyzed and subjected to two-dimensional electrophoresis and chromatography, producing two-dimensional peptide maps in order to further compare the primary structure of the proteins. Each inhibitor will be tested against such standard proteolytic enzymes as trypsin, collagenase, papain and elastase using hemoglobin and other pure proteins as substrates to obtain a general idea of its inhibitory spectrum. Each inhibitor will also be tested for its effect on enzymes derived from a given species' polymorphonuclear leukocytes and macrophages, using as substrates material obtained from fractionated lung tissue to more closely approximate its actual effect in the intact animal.

2. The Role of Cigarette Smoke and Nitrogen Dioxide in Determining Proteinase:Antiproteinase Interaction

a. Effect of Smoking or Nitrogen Dioxide on Proteinases from Lung Macrophages, Lung Tissue, and Polymorphonuclear Leukocytes.

The response of lung tissue to both acute and chronic treatments with cigarette smoke and nitrogen dioxide, have been amply described micro- and macroscopically. Several laboratories (10,16) have supplied data detailing the numerous changes resulting from *in vivo* exposure of pulmonary tissue to these agents but none has sought to determine their effects on the interaction between autogenous proteinases and antiproteases.

Populations of lung macrophages will be obtained by lavage (4). PMNs will be obtained from whole blood by the usual procedures used in our

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laboratory (24). Lung cells will be prepared by mincing lung tissue and homogenizing in a Potter-Elvehjem homogenizer, disrupted by sonication, and made into acetone powders. The powder will be extracted using water, 1 M NaCl, and 8 M urea to effect an initial fractionation corresponding roughly to acid, neutral, and alkaline proteinases. Work currently in progress in our laboratory using the PMN material from dog blood indicates: 1) the water extract is much richer in acidic proteinase (catheptic) activity than the combined neutral and alkaline activities; 2) the acidic activity is much less in the sodium chloride extract and essentially zero in the urea extract; 3) the sodium chloride extract has the greatest amount of alkaline and neutral activities; 4) the neutral and slightly acidic (pH 5) activities of the urea extract are more significant than either the acidic or alkaline proteolytic activities; 5) elastolytic activity, using Boc-L-alanine ester as substrate, is significant only in the fraction of the urea extract subsequently precipitated between 20 and 40% saturated ammonium sulfate; 6) collagenase activity, using bovine achilles tendon as substrate was found in both the water and the sodium chloride extract, with minimal activity in the urea extract. These findings are important in that they 1) demonstrate an initial significant separation of the major proteolytic activities and 2) the two principal enzymatic activities involved in connective tissue destruction, i.e., elastase and collagenase, and implicated in causing disease in these tissues (25) are easily separated. Although routine elastase assays are performed using a synthetic substrate, the use of elastin-orcein has confirmed the fact that the urea fraction precipitated by 20-40% ammonium sulfate did indeed have elastolytic activity.

In order to define which of these activities are important in emphysema induction we are preparing various fractions of lung tissue for use as more specific substrates for these enzymes. Those enzymes capable of destroying tissue-derived substrates will be employed in those studies dealing with proteinase-antiproteinase interactions.

Macrophages will be washed from lungs of dogs exposed to cigarette smoke or nitrogen dioxide. The pollutant exposure will be performed using sealed chambers into which the dog is placed and the appropriate pollutant piped in. We will also use intubated, anesthetized dogs in which the pollutant will be directly instilled into the lungs. Acute treatment will consist of 1-6 hours exposure to cigarette smoke adjusted to give an amount equivalent to two packs for an average weight smoker; or exposure to nitrogen dioxide at 30 ppm. Chronic treatment using cigarette smoke adjusted to give an amount equivalent to one-half pack in one group and two packs in a second group; or exposure to nitrogen dioxide at 2 ppm for one group and 17 ppm for another group (26) for varying intervals of time over a 0-6 month period.

The proteolytic activity of the macrophages will be determined using standard protein and synthetic substrates and also the specific lung substrates described above. Proteolytic activity will be measured not only using acetone powders of sonicated cells, but also from supernatants of cells allowed to merely discharge their enzymes due to possible changes in intracellular stability. These determinations of proteolytic activity will be performed on cells from both normal and treated dogs since there are reports (27) that both smoking and nitrogen dioxide treatment cause an increase in pulmonary proteolytic activity. Such data should be important in ascertaining if certain pollutants can potentiate the development of emphysema via the mechanism of proteolytic degradation.

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b. The Effect of Smoking or Nitrogen Dioxide on Serum and Lung Anti-proteinase Levels and Activities.

Sera from dogs subjected to acute and chronic smoking or nitrogen dioxide schedules will be assayed for changes of antiproteolytic activities using both the antitryptic assay of (28) and the more specific enzyme-substrate systems described above. Inhibition of specific antiproteolytic activities will be followed using the agar gel electrophoretic technique of Ouchterlony (29), in order to demonstrate not only different levels, but also different inhibiting spectra of antiproteolytic agents. These data will, therefore, aid in determining if cigarette smoke or nitrogen dioxide affect the level or composition of the various serum anti-proteinases.

Lung antiproteinase isolated as described in an earlier section will be assayed to determine possible changes due to exposure to either cigarette smoke or nitrogen dioxide. The total amount of lung antiproteinase, the amount bound in the lung to proteinases and the ratio of these values will be determined to give a quantitative picture of the relationship of pollutants to antiproteinase activity. Qualitative differences will be sought for by redetermining the spectra of enzymes inhibited by lung antiproteinase after exposure to cigarette smoke or nitrogen dioxide and comparing these to normal values.

c. Effect of Cigarette Smoke or Nitrogen Dioxide on Induction of Proteinase-Induced Emphysema.

Emphysema induction will be studied both in vivo and in vitro. Initially, our studies using PMN homogenates to produce emphysema will be extended to dogs previously subjected to various regimens of smoking or exposure to nitrogen dioxide as described in the preceding section. In our acute in vivo studies dogs exposed to cigarette smoke or nitrogen dioxide will be treated with various amounts and types of leukocyte-derived proteinase, sacrificed, and the severity of emphysematous lesions compared to those of untreated dogs. Chronic studies, as described previously, will also be performed prior to proteinase treatment to measure the short and long term effects of exposure to these agents on the ease of emphysema-induction using leukocyte proteinases.

In vitro studies will be carried out by removing lungs from dogs exposed to cigarette smoke or nitrogen dioxide and the isolated lobes used as test organs for proteinase studies. This method is not only more economical than whole animal studies but also emphasizes the effects of pollutants and proteinases on the lungs themselves, with a minimum of extra-pulmonary involvement due to circulatory transport of serum factors to act as antibodies, serum antitrypsin proteins or serum proteolytic enzymes. Previous work in the laboratory has demonstrated the feasibility of this technique for quantitating the amount of enzyme required to induce emphysema in the intact animal and should be of considerable utility in the program.

The significance of this program will lie in its determination of the role played by smoking and other airborne pollutants on the development

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of emphysema. By studying the effects of the above agents on both the serum and lung antiproteinases we hope to demonstrate which of the two is more important in conditions likely to cause emphysema. We will also better understand the role of proteinase-antiproteinase balance during induction of experimental emphysema. We hope to utilize the observations made in our model system in understanding the sequence of events occurring during the development of human pulmonary emphysema. In accomplishing this we believe that we shall be better able to describe those individuals most prone to emphysema development and, eventually, suggest a method of treatment.

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C. REFERENCES

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29. Duchterlony, O., Handbook of Immunodiffusion and Immuno-electrophoresis. Ann Arbor Science Publ. (1968).

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10. SPACE AND FACILITIES AVAILABLE:

The facilities available in the Biochemistry Laboratory for use during these investigations include:

1. Zeiss phase microscope
2. Sorvall centrifuge
3. Hitachi-Perkin Elmer spectrophotometer
4. Mettler balance
5. Refrigerator and freezer
6. Glassware for all the basic techniques described

The facilities available in the Cardiopulmonary Laboratories for use during these investigations include:

1. Eight-channel FM magnetic tape recorder with voice input.
2. Six-channel FM magnetic tape recorder with voice input.
3. Filtering and differentiator circuit.
4. Differential and vascular pressure transducers.
5. Animal body plethysmograph (modified small body respirator).
6. Spirometers.
7. Gas chromatograph for CO and N₂ analysis.
8. Godart CO Analyzer.
9. Godart CO₂ Analyzer.
10. Beckman O₂ Analyzer.
11. Blood gas and pH electrodes system with water bath and tonometer.
12. Pressure cycled respirator (Bird Mark VII).

The Research Laboratories have the following general facilities available:

1. Hotpack walk-in incubator
2. Walk-in cold room
3. RCA electron microscope
4. Dark room facilities

Animal boarding facilities occupy an adjoining building. An animal surgical suite is located there, contains a completely equipped operating facility and is maintained by a full-time staff. Standardization of dogs is practiced and during a three-week period of observation, testing and treatment prior to experimentation, pre-existing medical problems are eliminated.

11. ADDITIONAL FACILITIES REQUIRED:

NONE

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12. BIOGRAPHICAL SKETCHESA. PHILIP KIMBEL, M.D.

Head, Pulmonary Disease Section

Born:

REDACTEDRole in Project: Co-Principal InvestigatorEducation, Training, Honors

Temple University (Major in Sciences)

Temple University School of Medicine - M.D.

Internship, Albert Einstein Medical Center

Residency, Internal Medicine, Albert Einstein
Medical Center

USPHS Post-Doctoral Research Fellowship

Department of Physiology and Pharmacology

Graduate School of Medicine

University of Pennsylvania

REDACTEDProfessional ExperienceHead, Pulmonary Disease Section, Albert Einstein
Medical Center, Philadelphia, Pennsylvania

1961-

Professor of Medicine, Temple University Health
Sciences Center, School of Medicine,
Philadelphia, Pennsylvania

1971-

Associate Member, Research Laboratories, Albert
Einstein Medical Center, Philadelphia, Penna.

1968-

Associate Professor of Medicine, Temple University
Health Sciences Center School of Medicine.

1967-71

Associate in Medicine, Temple University Health
Sciences Center School of Medicine

1963-67

Instructor in Medicine, Temple University Health
Sciences Center School of Medicine

1960-63

Research Associate, Fels Research Institute,
Temple University School of Medicine andInstitute for Cancer Research (with Dr. S.
Weinhouse - Blood Glucose Metabolism). Part-time 1958-61Research Associate, Department of Physiology,
Graduate School of Medicine, University of
Pennsylvania (part-time). Worked with Dr. A. B.
Dubois and Dr. H. Linderholm in studies of
Pulmonary Capillary Blood Flow Simultaneously with
Cardiac Catheterization

1958-61

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B. GEORGE WEINBAUM, Ph.D.

Bioscientist, Pulmonary Disease Section.

Born: REDACTED

Role in Project: Co-Principal InvestigatorEducation, Training, Honors

University of Pennsylvania, Philadelphia, Pa. - A.B.

The Penna. State University, University Park,
Penna. - M.S.The Penna. State University, University Park,
Penna. - Ph.D.

Tokyo U. Inst. Appl. Micro, Japan - Post-doctoral

Albert Einstein Medical Center, Philadelphia, Pa.
Post-doctoralFulbright Research Scholar, Tokyo University
Career Development Awardee, Nat'l Inst. of Gen. Med.
SciencesProfessional Experience

I am presently an Associate Member in the Biochemistry Department. My research at Albert Einstein Medical Center has involved studies on animal and bacterial cell membrane structure and synthesis, enzyme biosynthesis and regulation, biosynthesis of naturally occurring nucleoside analogs in fungi, abnormal cell wall synthesis and characterization of the lipids of E. coli cell wall complexes. I am a Career Development Awardee.

From 1957-61, I was director of the Biochemical Section of the Pathology Department at Geisinger Medical Center, Danville, Penna. My research involved amino acid analogs and tissue culture cells. I spent one year (1959-60) as a Fulbright Research Scholar at the Institute of Applied Microbiology in Tokyo University. I was studying exoenzyme synthesis in B. subtilis.

I received my Ph.D. from Penna. State Univ. in 1957, having worked with Dr. M. F. Mallette on induced enzyme synthesis in E. coli.

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C. BRUCE SLOAN, Ph.D.

Research Associate

Born: **REDACTED**Role in Project: Research AssociateEducation, Training, Honors

Temple University - B.A.

Hahnemann Medical College - M.S.

Hahnemann Medical College - Ph.D.

Department of Pathology, Harvard Medical School

Post-doctoral

Department of Microbiology, Albert Einstein Medical

Center - Post-doctoral

Temple University - Dean's list

Hahnemann Medical College, U.S.P.H.S. Predoctoral
Fellow

REDACTED

REDACTEDProfessional Experience

I am presently a postdoctoral fellow in the Biochemistry Department, Pulmonary Disease Section, at Albert Einstein Medical Center. My research here has consisted of studies on proteolytic enzymes derived from dog polymorphonuclear leukocytes and macrophages. I have been attempting to assay, isolate and define the role of enzymes on the development of experimental emphysema using the dog as a model system.

From 1969-1971, I was a postdoctoral fellow in the Laboratory of Chemical Pathology, Department of Pathology, Harvard Medical School. My research was concerned with the role of genetics and the state of the antigen on cellular and humoral immune mechanisms.

I received my graduate training at Hahnemann Medical College, under Dr. Peter Stelos. My research was concerned with studies of the structure of immunoglobulin G, specific antibodies, and Bence-Jones proteins and employed enzymatic and chemical procedures for protein degradation and sequencing.

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13. PUBLICATIONS:

A. P. Kimbel, M.D.

Kaplan, A.S. and Kimbel, P.: Pulmonary Capillary Blood Flow Waves in Subjects with Abnormal Pulmonary Hemodynamics, Journal of Applied Physiology, 28:793 (1970).

Marco V., Mass, B., Meranze, D.R., Weinbaum, G. and Kimbel, P.: Induction of Experimental Emphysema in Dogs Using Leukocyte Homogenates, American Review of Respiratory Disease, 104:595 (1971).

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B. G. Weinbaum, Ph.D.

Okuda, S. and Weinbaum, G., Immunologic Cross-Reactivity of Escherichia coli B, Envelope Glycoproteins with Some Animal and Plant Cell Membrane Proteins, J. Immunol., 103:869 (1969).

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Kimbel, P., Mass, B., Ikeda, T. and Weinbaum, G.: Emphysema in Dogs Induced by Leukocyte Contents, Pulmonary Emphysema and Proteolysis, p.411. Edited by C. Mittman, Academic Press, Inc., New York (1972).

C. B. Sloan, Ph.D.

Sloan, B. The Extension of Thin Layer Electrophoresis on Cellulose to the Identification of DNS-amino acids. J. Chromatography 42:426 (1969).

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Sloan, B. and Gill, T.J. Synthetic Polypeptide Metabolism. IV. In Vivo and In Vitro Degradation of Poly (Glu⁵² Lys³³ Tyr¹⁵) in Highly Responding (ACI) and Poorly Responding (F344) Strains of Rats. Immunochemistry, 9:677 (1972).

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14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

George Weinbaum, Ph.D., Co-Principal Investigator

20

- 0 -

Philip Kimbel, M.D., Co-Principal Investigator

20

- 0 -

Bruce Sloan, Ph.D., Research Associate

50

REDACTED

Technical

Biochemistry Technician

100

8,000

Histology Technician

100

8,500

Fringe Benefits

4,580

Sub-Total for A

\$28,080

B. Consumable supplies (by major categories)

Dogs (approx. \$50 each)

3,000

Boarding of animals (\$1.25/day)

1,500

Rabbits (approx. \$6 each)

300

Glassware

1,000

Chemicals, Drugs, Stains, etc.

2,000

Sub-Total for B

\$ 7,800

C. Other expenses (itemize)

Travel to National Meetings

1,000

Books, Journals

300

Publication costs

500

Sub-Total for C

\$1,800

Running Total of A + B + C

\$37,680

D. Permanent equipment (itemize)

2 Isolation chambers for pollutant
exposure at \$1,000 each

2,000

Sub-Total for D

\$ 2,000

E. Indirect costs (15% of A+B+C)

E

5,652

Total request

\$45,332

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	REDACTED	7,800	1,800	1,000	5,955	\$46,655
Year 3		7,800	1,800	- 0 -	6,300	\$48,300

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE			
Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
<u>Etiology of Experimental Em- physema.</u>	N.I.H. - HE 13787-01	\$140,000	5/1/71 to 4/30/74
<u>Phospholipid Metabolism in Membrane Synthesis.</u>	N.I.H. - NS-07268-06	\$ 45,000	1/1/71 to 12/31/73
<u>Structure, Function and Synthesis of Cell Membranes</u>	N.I.H. - KO4-GM-07259- 04	\$17,500	7/1/69 to 6/30/74

PENDING OR PLANNED			
Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
<u>Proteinase:Antiproteinase Balance in the Lung and the Effect of Air-borne Pollutants</u>	National Tuberculosis Association (applied for 11/72)	\$22,678	5/1/73 to 4/30/74

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator is:

George Weinbaum, Ph.D.

Typed Name Philip Kimbel, M.D.

Signature *Philip Kimbel* Date 1/15/73

Telephone 215 DA 9 0700 363 or 463
Area Code Number Extension

Checks payable to

Jerome Baron, Vice-President for
Fiscal Affairs

Mailing address for checks

Albert Einstein Medical Center - N.D.
York & Tabor Roads, Phila., Pa. 19141

Responsible officer of institution

Typed Name Mr. Bertram Zimmerman

Title Acting General Director

Signature *Bertram Zimmerman* Date 1/25/73

Telephone 215 DA 9-0700 381
Area Code Number Extension

1003538728

EPIDEMIOLOGY

1003538729

ISSAR BELL

1003538730

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 13, 1973

CORRECTION
(Replaces memo dated February 2, 1973)

2

Grant Application No. 455B
(formerly No. 455AR1)

To: The committee comprising Drs. Gardner, Jacobson and
Sommers

Subject: Benjamin Bell, M.D., and Charles L. Rose, Ph.D., VA
Outpatient Clinic, Boston, Massachusetts
Continuation application No. 455B
"A Smoking Research Program in the Normative Aging
Study"

History

In view of the long and complicated history, we are again distributing "A History of Normative Aging Study Supported by the Council for Tobacco Research," prepared last year. The current grant is for \$64,000, without assurance of further support.

The request, also for \$64,000, is for "continuation", and therefore competes as a new application.

Documents Submitted (attached)

1. Application dated January 31, 1972, with summary progress report.
2. Manuscripts by Seltzer, Garvey and Bosse', presented at the Gerontological Society, December 1972.

(Also provided were various codes and questionnaires, which are not being forwarded at this time.)

Comment

Dr. Lisanti visits these grantees regularly.

Jm
F.W.N.

FWN:wg
Encls.

1003538731

Comm.

Dr. Gardner
Dr. Jacobson
Dr. Sommers

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application For Renewal of Research Grant

(Use extra pages as needed)

First Renewal ☒Second Renewal ☐

Date: January 31, 1973

1. Principal Investigator (give title and degrees): Benjamin Bell, M.D., and Charles L. Rose, Ph.D.

2. Institution & address: VA Outpatient Clinic, 17 Court Street, Boston, Mass 02108

3. Department(s) where research will be done or collaboration provided: Normative Aging Study (VA Special Purpose Research Laboratory)

4. Short title of study: A Smoking Research Program in the Normative Aging Study

5. Proposed renewal date: July 1, 1973 to June 30, 1974

6. How results to date have changed earlier specific research aims: The aims as set up in the original proposal have not changed; namely, to mobilize, coordinate and upgrade smoking research in the Normative Aging Study. The program exploits the availability of the Normative Aging population and the potential of its interdisciplinary and prospective design for smoking research. Since the ramifications of smoking effects are extensive, this program extends into virtually all aspects of the Study. For the period encompassed by this renewal request upward of 2,000 measures on 2,000 subjects for two examination times will be available.

7. How results to date have changed earlier working hypothesis: The results to date have been encouraging with respect to the above goals. The funds provided by CTR, though a small percentage of the total costs of the Normative Aging Study, have enabled a much larger scope of research than could otherwise have been possible.

1003538732

8. Any additional facilities now required? Describe briefly:

None

9. Any changes in personnel? Append biographical sketches of new key professional personnel: There have been no changes in personnel. During the period July 1, 1972 to January 31, 1973 the following professional personnel have been active:

Benjamin Bell, M.D. Principal Investigator
 Raymond Bossé, Ph.D. Program Coordinator
 Spencer Burney, M.D. Clinical Medicine and Biochemistry
 Paul Costa, Ph.D. Personality Research
 Albert Damon, M.D., Ph.D. Clinical Medicine and Anthropology
 James Fozard, Ph.D. Research Psychologist
 Arthur Garvey, Ph.D. Statistician and Data Manager
 Ronald Nuttall, Ph.D. Methodology
 Charles L. Rose, Ph.D. Research Sociologist, Co-principal Investigator
 Carl Seltzer, Ph.D. Anthropology

10. Append outline of experimental protocol for ensuing year.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent). The following manuscripts are appended:

1. Longitudinal Analysis of Smoking and Weight Change (Garvey)
2. Age and Interpersonnal Factors in Smoking Cessation (Bossé)
3. Death Rates and Smoking in the Elderly (Seltzer)

In addition, the following papers were published (copies have already been sent to CTR)

1. Significance of Functional Age for Research in Aging (Bell)
2. Strategy of Functional Age Research (Nuttall)
3. Measurement of Social Age (Rose)
4. Predicting Age from Body Measurements (Damon)
5. Auditory Functional Age (Bell)
6. Laboratory Functional Age (Burney)
7. Predicting Age from Abilities and Personality (Fozard)
8. Functional Age and Age-related Measures (Dempster)

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12. Summary progress report (append in standard form as separate document, unless recently submitted)

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited")

% time

Amount

Professional (give % time of investigator(s)
even if no salary requested)

Salaries

Professional

Bosse

85%

12,000

Nuttall

12%

3,500

Costa

8%

2,500

Consultant Fees

4,500

Technical

(5) Clerk typist

100%

6,800

(6) Computer Programmer

100%

9,800

(7) Research Assistant

50%

5,000

44,100

Fringe Benefits

10% of (1), (5), (6), & (7)

3360 - 10%

3360

Sub-Total for A 47,500

47,460

B. Consumable supplies (by major categories)

Office supplies and duplicating
Reprints and publication costs
Lab supplies

500

1,000

2,500

Sub-Total for B 4,000

C. Other expenses (itemize)

Data processing costs (computer time, key punching,
tape and disc rental, programming consultation)

10,000

travel for consultation and scientific meetings

1,500

Sub-Total for C 11,500

Running Total of A + B + C 63,000

D. Permanent equipment (itemize)

Storage cabinet for magnetic tapes

430

two (2) IBM card file cabinets @ \$285

570

Sub-Total for D 1,000

E none

E. Indirect costs (15% of A+B+C)

Total request 64,000

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14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Normative Aging Study	Research Service, VA Central Office, Washington, D.C.	\$196,000	July 1, 1973 to June 30, 1974
Support from funds of VA Outpatient Clinic, Boston including (1) physical facilities such as office space and equipment, telephone, maintenance, security, janitorial services utilities and clinic facilities, including laboratory x-ray and audiology and (2) personal services such as partial professional salaries of Study Director, Director of Clinical Medicine and Laboratory Research, Audiologist, radiologist, x-ray technicians, and clinical consultants.		200,000	

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
	none		

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Benjamin Bell, M.D.

Signature *Ben Bell* Date 1/29/73

Telephone 617-223-2052
Area Code Number Extension

Checks payable to

Normative Aging Study

Mailing address for check:

VA Outpatient Clinic

17 Court Street, Boston, Mass 02108

Responsible officer of institution

Typed Name Charles L. Rose, Ph.D.

Title Assistant Director, Normative Aging Study

Signature *Charles L. Rose* Date Jan 29, 1973

Telephone 617-223-2053
Area Code Number Extension

1003538735

Item 12. Summary Progress Report

(1) Spurred by the Special Smoking Research Program, we have set up a weekly scientific meeting. These meetings were materially aided by the presence of a CTR monitor. A smoking questionnaire has been completed and approved for use by the Bureau of Management & Budget in Washington. The questionnaire, which is appended, is currently being sent to the 2,000 Normative Aging subjects. A drinking questionnaire has been completed but not yet approved by the Bureau of Management & Budget. A lifestyle questionnaire, including sociological and personality items is in preparation.

(2) Two procedures have been instituted for measurement of CO, CO₂, and O₂, through analysis of expired air, and analysis of blood gases through the use of ear-lobe blood. The expired air analysis is being done in the laboratories of the Harvard School of Public Health. The feasibility study of these procedures are now in their final stages. If feasible, and at this writing it would appear to be so, the data will be related to smoking history, and for smokers will be taken experimentally before and after smoking. Also on a volunteer basis, nonsmokers will participate in the experiment. This will provide for the first time the distribution of the measures of these gases in a large series of normals, providing norms for healthy individuals that do not yet exist. Also the effect of smoking on the level of these gases will be determined.

(3) A pilot project has been started with the Cardiology Service (Dr. Haber) of the Massachusetts General Hospital to differentiate hypertensives through blood and urine measurement, renin, aldosterone, cortisol, and mineral corticoids. If this study is successful, we can determine if there is a relationship between smoking and these measures which are indices of stress and part of the hypertensive state as hypothesized by the MGH group.

(4) Blackburn coding has been completed on 2,000 EKG's through a contract with the School of Public Health of the University of Minnesota. The Blackburn code is a descriptive method for assessing the EKG and gives us an objective tool for assessing cardiac status and its relationship to smoking habits, as well as other health measures.

(5) A symposium on Smoking and Age was organized for the 25th Annual Scientific meeting of the Gerontological Society of December 19, 1972 at San Juan. Three of the papers came from the Normative Aging Study (Drs. Seltzer, Bosse', and Garvey). A copy of the program is appended. The motif of the symposium was that smoking be investigated not merely for its relationship to mortality and morbidity but also for its relationship to normal aging processes. The latter, of course, is the emphasis in the Normative Aging Study and represents a unique or little used approach in smoking research.

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Item 10. Outline and Proposed Research, July 1, 1973 to June 31, 1974

- (1) We will develop questionnaires on drug and pill taking, life styles and environment, and personality. Data analysis will be completed on these instruments in addition to the smoking and drinking questionnaires which have already been completed. Data on drinking, drug and pill taking will be collected in addition to smoking since they all have in common a relationship to psychological or physiological dependence. Also, they contribute or may contribute to change in health status and it would be important to investigate the relative importance of each in this regard.
- (2) Relationship between smoking and clinical status in tooth decay and periodontal tissue will be studied. The dental data are now being merged with Normative Aging data in one data file. Data management and analysis will be carried out primarily by the statistical and computer programming personnel of the Normative Aging Study.
- (3) A longitudinal analysis over two examination times 5 years apart will be carried out on changes in smoking habits in relation to changes in blood pressure, cholesterol, triglycerides and pulmonary function. This will allow us to examine the effect of smoking habits on change over time in these physiological variables.
- (4) Personality type A and B assessments will be carried out with Jenkins' objective instrument (see appended: Jenkins Activity Survey of Health Prediction). Since these personality types are said to be related to coronary artery disease, we will also examine their relationship to smoking. This will determine whether the work of Roseman and Friedman (The Western Collaborative Study) at the Mt. Zion Hospital in San Francisco relating coronary artery disease to personality, utilizing a clinical interview for assessing personality, is replicable with the objective questionnaire of the Jenkins type.
- (5) We will study the association between amount of coffee and tea drinking and selected physiological and behavioral variables. Insofar as our data will allow, we will study the relationship of coffee and tea drinking with mortality and morbidity. This will permit us to determine whether the results obtained by the Drug Surveillance Program of the Boston University Medical Center using sick patients (Lancet, 12-16-72) apply to our normal series.
- (6) Subjects who have died will be compared to surviving age peers with respect to smoking, cholesterol, social class, parents' age at death and personality traits, in order to determine those variables which best discriminate between them either singly or in combination.
- (7) We will be conducting research which relates personality traits to measures of job satisfaction and family cohesion across three age groups. Specifically, we will investigate the relationship of the variables to smoking habits as well as to age. Preliminary analysis shows that the anxiety prone and those who have more contacts with kin do smoke more. We hope that a more detailed analysis will clarify these findings. Presentation of the data is scheduled at a Symposium on Personality, Aging and Social Systems at the American Psychological Association annual meeting, Toronto, August 1, 1973.

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(8) Developments in data management will include creation of two master files: one for data collected during the first 5 year cycle, and a second which will include the second cycle. This will speed longitudinal analysis by increasing efficiency in retrieval of data from the computerized files.

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#870 EYSENCK

1003538739

November 22, 1972

1

Grant application No. 870

To: The committee comprising Drs. Gardner, Huebner, and Sommers

Subject: Professor H. J. Eysenck, Ph.D., D.Sc., Institute of Psychiatry,
London
New application No. 870
"Inheritance of the Smoking Habit"
(N.B. This application in draft form was sent to the
Planning Committee July 24, 1972.)

History

Dr. Eysenck, who participated in the St. Martins', January 1972 meeting, has been in correspondence with Dr. Hockett. The remaining history of this application will be seen from the accompanying documents.

Application No. 870 requests 8,625 pounds, plus two additional years.

Documents Submitted (attached)

1. Application dated July 7, 1972
2. "The Inheritance of the Smoking Habit", (six pages, plus two pages of references)
3. Letter to Dr. Hockett dated July 6, 1972
4. Letter to Dr. Hockett dated November 3, 1972
5. Letter from Dr. Seltzer to Dr. Hockett, dated October 30, 1972, presenting an evaluation of the proposal. (We are asking Dr. Eysenck the questions raised by Dr. Seltzer).

Comments

We have a copy of "Personality and the Maintenance of the Smoking Habit" presented by Eysenck at the St. Martins' conference in January. A copy of this voluminous document will be forwarded if you so request.

On December 8, 1972 the Planning Committee will be asked to consider Eysenck's request (top paragraph, page 2 of his November 3, 1972 letter) for approximately 2,000 pounds for interim support of two people until April 1973. (We are asking the applicant for details of this request).

F.W.N.
F.W.N.

FWN:WG

Encl.

cc: Planning Committee

1003538740

870

HARVARD UNIVERSITY
SCHOOL OF PUBLIC HEALTH

TEL. (617) 734-3300
(617) 734-3311 (AFTER HOURS)
CABLE ADDRESS: NUTHARV, BOSTON

DEPARTMENT OF NUTRITION
665 HUNTINGTON AVENUE
BOSTON, MASSACHUSETTS 02115

October 30, 1972

Dr. Robert C. Hockett
The Council for Tobacco Research-USA
110 East 59th Street
New York, N. Y.

Dear Bob:

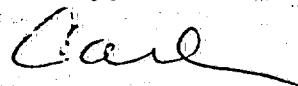
I have finally completed analyzing the Eysenck proposal "The Inheritance of the Smoking Habit."

In my opinion, this investigation is well-worth doing and I trust it will be approved. In following the procedures of the Jinks and Fulker methodology it should produce interesting and important information. My only reservations have to do with the sample of twins to be obtained. Does Eysenck has assurance of access to the twin registries? Does he have some assurance of cooperation from these twins? After all, he needs fair size numbers. Can he obtain adequate numbers of foster children?

My questions have to do with the practical aspects of the proposal, not the methodological and theoretical aspects which appear feasible.

I am sorry for the delay, but the Jinks and Fulker article is extraordinarily formidable. I wish I could say I understood it completely.

Sincerely,



Carl C. Seltzer, Ph.D.

CCS:ek

1003538741

Applic. #870

NATIONAL RESEARCH COUNCIL

NATIONAL ACADEMY OF SCIENCES NATIONAL ACADEMY OF ENGINEERING

2101 CONSTITUTION AVENUE WASHINGTON, D.C. 20418

DIVISION OF MEDICAL SCIENCES
FOLLOW-UP AGENCY

29 January 1973

Dr. Frederic W. Nordsiek
Associate Scientific Director
The Council for Tobacco Research
10th Floor
110 East 59th Street
New York, New York 10022

Re: Your Grant Application No. 870

Dear Dr. Nordsiek:

I reviewed the proposal you sent me for a study on the inheritance of the smoking habit. Unfortunately, while the proposal is by a worker well known in this area of research, and is quite ambitious, it is presented in a very summary fashion. I can only be skeptical of the feasibility of the proposed undertaking, but find very little in the proposal to help me overcome my skepticism.

The question to be investigated, that is the role of genetic factors in smoking behavior, is one that is reasonable and, in my own opinion, very much worth answering. The proposal tells me that Fisher, and Friberg et al., and others, whose work I do not know, have done studies on this subject. There is a general methodologic evaluation of these studies and an interpretation that, "the evidence is suggestive but not conclusive and (that) it leaves many questions unanswered". However, there is no statement about the specific nature of this evidence and how it relates to the objectives of the proposed work.

Certainly one of the fundamental elements of the work is the specific nature of the model that will be used. What is the hypothetical structure of this model, what assumptions must be made before the collected data can be applied to it, and what are the criteria by which the model will be judged applicable? These questions are dismissed by a reference to a general textbook.

It is indicated that certain assumptions, such as equality of environmental components for twins, will be assessed after the analysis by the test of goodness of fit of the model. This may not be an effective approach. Some very unreasonable models might appear to fit the data by coincidence or because the sample may not have sufficient power to discriminate between different models. The validity of assumptions could be evaluated to some extent before the model is applied and perhaps the model could even be made consistent with the results of such evaluations. Unless more is said about the model these issues remain unclear.

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It is indicated that "the choice of the sample to be tested is of course crucial to the whole investigation", and of course it is. This statement is followed by a listing of the numbers of hypothetically desirable relative pairs that include mother, father, daughter, son, sibs, unrelated males and females, foster parents, and foster children, living together and living apart; and it is stated that, "something like 1,000 families should provide enough independent pairs to satisfy the requirements of the design". The method for obtaining this sample is indicated as the existence of a twin register of about 1,000 pairs of twins. Nothing is mentioned about the procedure used in compiling this register. Who are these twins, what are their ages, do they represent any definable group, what is their distribution by sex and zygosity, is there any evidence at all that they will even approximately provide the numbers of "independent pairs" specified in the listing?

In the proposal there is no conceptualization of the problem to be investigated. It would be reassuring to find some recognition of the idea, that the genetic component of smoking behavior can be entirely suppressed by the environment. It can find expression only if the environment provides access to tobacco products and continues to permit their use. This means that it might be especially useful in these studies to be concerned with economic factors such as income in relation to tax on tobacco; social factors, such as esthetic, ethical, or religious persuasions; and factors that encourage cessation of smoking, such as health education campaigns. The action of such factors may in turn be partly genetically determined. If the effect of these related factors could be described and isolated in this study and distinguished from other aspects of the genetics of smoking behavior, then the study would indeed make a most important contribution. However, there is little in the proposal to indicate that the study might accomplish any important objectives except that it emanates from a group that has been productive in the past.

Sincerely,

Zdenek Hrubec
Zdenek Hrubec

ZH/mb

cc (by CTR)
Mrs. Gardner
Hockoff
Huber
Jacobson
Johnson

1003538743

THE JACKSON LABORATORY
BAR HARBOR, MAINE

MEMORANDUM

3 January 1973

To: DR. F. W. NORDSIEK, ASSOCIATE SCIENTIFIC DIRECTOR, CTR
From: H. MEIER *H.M.*
Subj: APPLICATION NO. 870: "THE INHERITANCE OF THE SMOKING HABIT"
BY EYSENCK

Although I have carefully read the proposal, due to a number of current pressures I took the liberty of asking one of my colleagues to confidentially review it. He is a behavior geneticist, and knows a number of the people involved or cited in the proposal; he is also familiar with some of the statistical procedures proposed. Here is his verbatim report:

I have a sweet and sour reaction to this research proposal.

Sour:

Eysenck seeks to be controversial. This isn't bad, just a fact. He plays to the press.

'Gene action' doesn't really mean gene action. It's simply a term that is attached to certain irregularities or regularities of the statistical model used in the genetic analysis. Models change.

"...it is proposed to use their (Jinks and Fulker) model and statistical treatment for the purposes of analysing the data to be obtained in this study." The real rationale for the proposal rests on extensions of Mather's biometrical genetic models by Jinks and Fulker and the justification for his attempting to sell it rests on all previous studies not either using the 'new' technique or in not studying all possible human pair relationships. I consider this quite weak.

"...it would be out of place to go into them (the general principles of the technique) in detail here." Truly said. Eysenck does not know them.

"Eaves will act as Consultant to this project." Yes, Eaves is the one who will do the entire project. Eysenck will attach his name to the published results.

Sweet:

Eysenck is indeed skilled in developing questionnaires. Mike Curtis probably has a book he wrote on how to test your own IQ.

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The personality variables will be judged according to factor analysis constructs that he, himself, defined. These constructs are simply names given to vectors that seem to make sense to the investigator. The dimensions are extraversion-introversion and neuroticism-stability. If anyone can interpret the smoking data in terms of these vectors, Eysenck can.

The considerations of sampling are interesting and good...again Eaves.

The use of virtually all possible human relationships in the design of the experiment is a good feature, although taxing. I can think of only one human pair relationship not considered in this list...that of son-godfather.

Summary feelings:

A great quantity of interesting data will be collected in the proposed study. The expertise of Jinks and his co-workers lie at the crux of the proposal. It's a pity, I feel, that Eaves, himself, didn't author the grant request.

A decision to award the proposal should be based on:

(1) the desire of the agency to take the bait--overtly or covertly--in the hopes of injecting more controversy into the resolution of the question.

(2) the budget (unspecified in the narrative). If it's large, the proposal should be scrutinized by several others. If small, it can be passed on to the consumer.

HM:tg

cc (by TR)

Drs. Gardner

Horkitt

Hubner

Sommers

Copy sent
1/5/13

1003538745

Applic #870

29 January 1973

Dr. Frederic W. Nordsiek
Associate Scientific Director
The Council for Tobacco Research-U.S.A., Inc.
110 East 59th Street
New York, New York 10022

Dear Dr. Nordsiek:

Here is my evaluation of Professor Eysenck's proposal for research on "The Inheritance of the Smoking Habit". Clearly my reaction is totally negative and for a number of reasons.

Support of this research is not recommended. It will produce no useful information and make no contribution to knowledge.

Both individuals and families are genetically different and will therefore respond to the circumstances and events in their environment in different ways. It is to be expected that many, if not most, likes and dislikes (habits?) will show some correlation with degree of genetic similarity, i.e., on average genetically more similar individuals (monozygotic siblings) will behave more alike than genetically less similar individuals (unrelated individuals). That is all that a heritability greater than zero tells us, i.e., what we already know.

As R. A. Fisher pointed out long ago "the so-called coefficient of heritability, which I regard as one of those unfortunate short-cuts which have emerged in biometry for lack of a more thorough analysis of the data" (British Agricultural Bulletin, 1951, 4, p. 217).

A human heritability estimate is both deceptive and trivial. It misleads too many unsophisticated and gullible people into believing they have acquired a profound insight, when in reality all it provides is a restatement of previously existing knowledge -- as in Moliere's Le Bourgeois Gentilhomme when M. Jourdain acquires the deep insight "Good Heavens! For more than forty years I have been speaking prose without knowing it."

There are serious contradictions in the model being proposed. It assumes additivity of alleles and loci and that they can be represented by values on a quantitative scale. Take, for example, the simplest conceivable polyallelic case of three alleles at one locus, e.g. A_1 , A_2 , A_3 and give them values 0, 1, 2

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Dr. Frederic W. Nordsiek

29 January 1973

Page 2

respectively on a scale. Notice what this means: if there were two populations each containing just two alleles of equal frequency, one with only alleles A_1 and A_2 and the other with A_1 and A_3 , the second population would have a much larger genetic variance than the first -- a perfectly nonsensical counterintuitive result. It is strictly an artifact of the proposed inappropriate model. It is exactly for this reason that the brilliant population geneticist Richard Lewontin (1972)* abandoned such a model in favor of the information or uncertainty measure in order to describe within and between population diversity with respect to known loci.

It is precisely such inconsistent genetic variances that lie at the foundation of the worthless heritability measure.

There is also the fact that over several decades Eysenck has not been meticulous about the quality, accuracy or rigor of what he publishes. I have no confidence in any publication that bears his name. See:

Charles Hanley and Milton Rokeach 1956. Care and carelessness in psychology. Psychological Bulletin, 53, p. 183 and references therein.

Richard Christie 1956. Some abuses of psychology. Psychological Bulletin, 53, p. 439 and references therein.

David T. Lykken 1959. Turbulent complication. Contemporary Psychology, 4, p. 377.

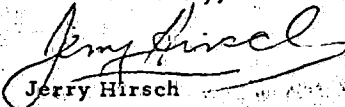
L. H. Storms and J. J. Segal 1958. Eysenck's personality theory with special reference to 'The Dynamics of Anxiety and Hysteria'. British Journal of Medical Psychology, 31, p. 228.

The Sunday Times of London, 20 June 1971. The Fallibility of H. J. Eysenck.

Also see H. J. Eysenck. Genetics and Personality.

In J. M. Thoday and A. S. Parkes (Eds.) Genetic and Environmental Influences on Behaviour. Edinburgh: Oliver and Boyd 1968 on p. 163 of that book he quotes me out of context so that the words used have exactly the opposite meaning to that which they have in my immediate text and throughout the article from which he "ripped them off".

Yours sincerely,


Jerry Hirsch

JH/br

*R. C. Lewontin. The apportionment of human diversity. In Theo. Dobzhansky, Max K. Hecht and William C. Steere (Eds.) Evolutionary Biology. New York: Appleton-Century-Crofts, 1972.

cc (by (TR)): Drs. Gardner, Hocke, Hrobman, Jacobson, Johnson

1003538747



DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
HEALTH SERVICES AND MENTAL HEALTH ADMINISTRATION

January 24, 1973

NATIONAL INSTITUTE OF MENTAL HEALTH
MENTAL HEALTH INTRAMURAL
RESEARCH PROGRAM
9000 ROCKVILLE PIKE
BETHESDA, MARYLAND 20014
AREA CODE 301 TEL: 656-4000

Frederic W. Nordsiek, Ph.D.
Council for Tobacco Research - U.S.A., Inc.
110 E. 59th Street - 10th Floor
New York, New York 10022

Dear Dr. Nordsiek:

I find this research proposal methodologically sophisticated but conceptually naive. The latter finding admittedly reflects my own prejudices about the inheritance of complicated behavior and about the pitfalls of twin studies. My reservations are based also on the impression that Professor Eysenck will, as he has sometimes done in the past, be so bent on reaching a firm conclusion from his study that his interpretation of results may go far beyond what I would consider justifiable.

Despite these reservations, I am inclined to recommend approval of the research grant. First, Professor Eysenck has a very thorough grasp of methods of psychological testing and of statistical analysis, and his consultant in genetics, L. J. Eaves, has made important contributions to the statistical analysis of twin data. Consequently, the proposed study is almost certain to yield some excellent new data on "twin heritability" of psychological variables.

Second, the research is a logical, if not a necessary, inquiry into the meaning of the now securely established association between smoking and certain diseases. As long as that association lacks a complete causal explanation, it can be attributed to some antecedent variable, either genetic or environmental, that is independently responsible for the smoking habit and associated diseases. Other studies are examining the causes of disease; this study will expose the link, if any, between heredity and the smoking habit.

At this point the deficiencies of the twin method will be limiting, because even if the smoking habit can be tied to psychological traits, these traits will not be conclusively assignable to either heredity or the early childhood environment. For the present purposes, however, that is not important. If the results should be negative, they could effectively exclude heredity as an explanation of the association between smoking and disease. That would not establish

1003538748

page 2 - Dr. Nordsiek

the causal role of smoking, but it would make it more probable.

Regarding the proposed budget, I find no evident defects, but I would disclaim any expertise in this aspect of research planning.

Sincerely yours,

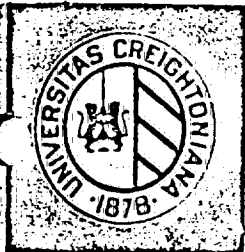
Gordon Allen

Gordon Allen, M.D.
Laboratory of Socio-environmental
Studies, NIMH

cc. Drs. Gardner
Huebner
Sommer
Hockett

(also Mar. '73 agenda book)

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CREIGHTON UNIVERSITY

OMAHA, NEBRASKA 68131

SCHOOL OF MEDICINE
DEPARTMENT OF PREVENTIVE MEDICINE
AND PUBLIC HEALTH

December 20, 1972

Frederic W. Nordsiek, Ph.D.
Associate Scientific Director
Council for Tobacco Research--
U.S.A., Inc.
110 East 59th Street
New York, New York 10022

Dear Dr. Nordsiek:

Professor H. J. Eysenck is an established and reknowned research psychologist who has, personally and with associates, developed numerous psychological testing devices. He has pioneered studies relating constitutional and personality variables. I would expect any study involving Professor Eysenck to produce useful data and interesting interpretations. Regarding the specific proposal entitled "The Inheritance of the Smoking Habit", I am intrigued by the investigator's intended contributions "... not only to partition the variation in the population into environmental and genetic causes, but also to provide information on the type of gene action involved. . . ." It is not clear to me, however, how the data to be obtained in Professor Eysenck's proposal study, could yield such information. Therefore, based on the information in the application, I am unable to render any specific judgement other than to conclude that the given proposal does not include information that would be sufficient for me to evaluate objectively.

The phenomenon of "heritability", which seems to be basic to Professor Eysenck's project, involves considerable controversy. Many geneticists consider that concept meaningless for any human behavioral or personality characteristic. The concept was originally based on studies of experimental organisms in which both genotype and environment could be controlled and duplicated. Any modification in genotype or in environment alters "heritability". Perhaps, in this regard, you might be interested in seeking the reaction of a reknowned investigator in human behavior genetics, who has previously been very critical of Professor Eysenck's interpretations: Professor Jerry Hirsch, Departments of Psychology and Zoology, University of Illinois, Urbana, Illinois.

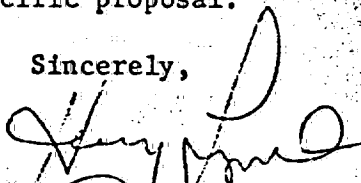
I hope that the Council for Tobacco Research--U.S.A., Inc., will consider this proposal as meriting appropriate revision with specific deliberation pertaining to rectifying the several points mentioned. Dr. Eysenck should

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read some of the material published by Lillienfeld and Tokhuata and perhaps this will help him to understand some of the genetic investigations already accomplished on lung cancer.

I am very doubtful, however, that his specifically cited objectives could be met; and I don't believe that the given information is sufficient for objective evaluation of his specific proposal.

Sincerely,



Henry T. Lynch, M.D.
Professor and Chairman
Department of Preventive Medicine
& Public Health

HTL/sah

cc (by (TR)

Drs. Gardner

Hockett

Harber

Jacobson

Sommers

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THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

Application For Research Grant

JUL 27 1972

Date: July 7th 1972

1. Name of Investigator(s): (include Title and Degrees)

Professor H.J. Eysenck, Ph.D., D.Sc.

2. Institution &

Address:

/Department of Psychology
/Institute of Psychiatry
. DeCrespigny Park, London, SE5 8AF
/England

3. Short Title of Project:

The Inheritance of the Smoking Habit

4. Proposed Starting Date:

January 1st 1973

5. Anticipated Duration of this Specific Study:

3 years

6. Brief Description of Objectives or Specific Aims:

The objective of the proposed study is to investigate the heritability of the tobacco smoking habit. There have been several attempts in the past to do this, briefly reviewed in Section 8; these fail to come up to modern methodological requirements and cannot be interpreted in terms of an acceptable genetic model. The proposed study seeks not only to partition the variation in the population into environmental and genetic causes, but also to provide information on the type of gene action involved, and to permit a statistical test of the adequacy of the assumptions underlying the genetical model to be used. An additional objective of the study is to discover the degree to which the genetic and the environmental parts of the individual's smoking behaviour are determined by personality factors, and to investigate in turn the extent to which these are inherited.

7. Give a Brief Statement of your Working Hypothesis:

The hypothesis to be investigated states that a significant proportion of the total variants of smoking behaviour is determined by hereditary causes.

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8. Details of Experimental Design and Procedures: (Attach Separate Pages)

See Attached Paper

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

Departmental facilities include a twin register which will be used extensively for this study, and card punching and computer facilities, including direct access to University Computer facilities.

10. Additional Requirements:**11. Biographical sketches of all principal and professional personnel (append)**

No final decision has yet been made about the professional assistants to be appointed, and consequently the only biographical sketch included is that of the principal investigator.

12. List of publications: (Five most recent as pertinent) (append)

Five relevant papers are quoted in the

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13. Budget: (1st year)

3.

A. Salaries (Personnel by names)

Professional

Psychologist

Social Worker

Consultant (Dr. L. Eaves)

% time

Amount

£

100%

2,500

100%

2,200

10%

500

Technical

Secretary

100%

1,500

Sub-Total

B. Consumable Supplies (list by categories)

Tests

300

Sub-Total

7,000

C. Other Expenses (itemize)

Postage

Travel

300

200

Sub-Total

7,500

D. Permanent Equipment (itemize)

E. Overhead (15% of A+B+C)

Total

1,125

8,625

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	£ 7,000		200		1,080	8,280
Year 3	£ 7,300		200		1,125	8,625

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature

Director of Project H. J. Eysenck

Signature

Business Officer of the Institution

Telephone

Telephone

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O.K.
R.E.H.

Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current

Title of Project

Source

Amount

Duration

Pending

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THE INHERITANCE OF THE SMOKING HABIT

A. The Problem

The investigation here described was planned in order to investigate the heritability of the tobacco smoking habit; the aim is not only to enable the variation in the population to be partitioned into environmental and genetic causes but also to provide information on the type of gene action involved, and to permit a statistical test of the adequacy of the assumptions underlying the genetical model to be used. The general problem of the genetic determination of smoking is clearly central to such theories as Fisher's, which attempt to relate the statistical correlation between smoking and disease (e.g. lung cancer) to a genetic cause responsible for both smoking and disease. A theory of this type has been elaborated by the writer (Eysenck, 1965), by relating smoking to personality type, and by showing that personality type was also related to the development of cancer (Kissen and Eysenck, 1962); it will consequently also be part of the present study to investigate simultaneously with the genetic determination of smoking the genetic determination of personality type.

B. Previous Work

Several studies have been done in this field since Fisher's (1958) early work; we may mention the work of Friberg et al. (1959), Todd and Mason (1959), Raaschou-Nielsen (1960), and Conterio and Chiarelli (1962). These studies have employed the classical method of using monozygotic twins reared together and dizygotic twins reared together; either intraclass correlations are calculated for both groups of twins, and an index of heritability obtained, or the ratio

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of the within-pair variance of the DZ twins to that for MZ twins is used as an F-test of the importance of genetic factors in determining variation. There are two primary deficiencies in these classical studies; they fail to take account of variation due to genetic and environmental influences acting between families, and they can provide no conclusive information about the type of gene action involved. More informative is a study done here at the Institute by Shields (1962), in which an additional sample of MZ twins reared apart was used, but the statistical treatment fell short of modern methods, and cigarette and pipe smoking were confused by adding these two different methods of consumption into one single index. It may be concluded that the evidence is suggestive but not conclusive, and that it leaves many questions unanswered, particularly those relating to gene action. Even with respect to the purely genetic question of the degree to which smoking is determined by hereditary factors no clear answer can be given in view of the neglect of the between-family variance.

C. Methodology: Statistical

The approach of biometrical genetics developed by Fisher and, following him, by Mather (1949) provides the basis for a genetically meaningful evaluation of the principal components of variation which is readily extended to the analysis of continuous variation in the human population. The application of biometrical genetics to human psychogenetics has been the subject of a study by Jinks and Fulker (1969), and it is proposed to use their model and statistical treatment for the purposes of analysing the data to be obtained in this study. The general principles underlying this approach will be found in Mather and Jinks (1971), and it would be out of place to go into them

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in detail here. The writer has worked with Professor Jinks in the past, and Dr. L. Eaves, a member of his team, has agreed to act as Consultant to this project and has been helpful in making suggestions relating to the choice of a suitable sample (cf. Section E, *infra*). Here, it may be useful merely to note the main advantages of this new approach.

1. The new approach begins by postulating a proper genetic model and incorporates tests for genotype-environment interaction and genotype-environment correlation. The randomness of mating and the equality of environmental components for twins, sibling and half-siblings are assessed after the analysis by the test of goodness of fit of the model.
2. The model provides an assessment of the relative importance of dominance.
3. The model provides a meaningful assessment of between-family variation and of interaction. These are important advantages, compared with the paucity of information that could be obtained by means of the classical method of twin comparison.

D. Methodology: Experimental

It is intended to obtain information by questionnaire on each person's smoking habits both present and past, including questions on inhaling, cigarette, cigar and pipe smoking, and in particular of course the amount of tobacco consumed. The questionnaire to be used would be modelled on that used in our past enquiries (Eysenck et al., 1960; Eysenck, 1963). A second questionnaire would deal with the occasions on which a person was most likely to smoke; the

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questionnaire to be used is the one prepared and used in our laboratory by Frith (1971), which has been shown by factor analysis to measure two main tendencies, i.e. to smoke under conditions of boredom and fatigue, or to smoke under conditions of stress and anxiety. A third questionnaire would deal with personality variables, particularly extraversion-introversion, which has in the past been shown many times to be related to smoking, and neuroticism-stability which has been shown to correlate with smoking only in women (Eysenck, 1973). In addition, the questionnaire would contain a Lie Scale, to test for test-taking attitude and dissimulation (Michaelis and Eysenck, 1971), and another mental health scale which in preliminary work we have found to correlate with smoking (unpublished). The total questionnaire would be printed, with instructions, and laid out to make transfer of results to computer cards easy; all analyses would be carried out on the computer, using specially written programmes.

E. Methodology: Sampling

The choice of the sample to be tested is of course crucial to the whole investigation as the parameters of the model require to be measured and tested for fit by reference to particular types of family relations. It is possible to deduce from the model quantitative rules which maximise the information obtained for each test carried out (Eaves, 1969), and Dr. Eaves has kindly worked out an optimum set of pairs, with optimum numbers for each set of pairs, to use in the proposed investigation. The list is as follows:

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<u>PAIRS</u>	<u>NUMBER OF PAIRS</u>
Father-son together	200
Mother-daughter together	150
Father-daughter together	100
Mother-son together	100
Male sibs together	150
Male sibs apart	100
Female sibs together	150
Female sibs apart	100
Unlike sex sibs together	150
Unlike sex sibs apart	100
Male MZ together	100
Female MZ together	100
Unrelated males together	150
Unrelated females together	150
Foster-father - Foster-son	150
Foster-father - Foster-daughter	150
Foster-mother - Foster-son	150
Foster-mother - Foster-daughter	150

Professor Jinks has suggested that, in addition, data be obtained from the parents of sib pairs, and from the relatives of parent-child pairs. This would provide a "second sample" from which data could be selected if necessary to augment particular statistics for further analysis. Something like 1,000 families should provide enough independent pairs to satisfy the requirements of the design and provide supplementary data. It will be clear that these are "ideal"

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requirements; it is unlikely that we will be able to obtain all the needed data, or that we will be able to obtain them in the precise proportions laid down. Fortunately the design is not rigid, in that some of the information is redundant; the skill in the analysis lies of course in making optimum use of what information is in fact available. It is for this reason that Dr. Eaves would be retained as Consultant.

Partly in preparation for this study, we have inaugurated a twin register which, at the moment, contains approximately 1,000 pairs of twins; these will provide us with the beginning of our sample, together with their sibs, parents, grandparents and other relatives. Foster children will of course have to be traced separately, through adoption agencies.

F. Budget

The work requires two Psychologists, or one Psychologist and one Social Worker, to trace and contact the subjects for the investigation; it is too early to be able to name the people involved. In addition, one Secretary would be required. Dr. L. Eaves would act as Consultant. In addition, there is provision for postage and printing, and for travel as not all the subjects live within the Greater London area. * Details are given on the official form. It is proposed that the study should continue for three years in all. I would personally direct the project and be responsible for the analysis and the writing up of the results, but my services do not form part of the budget.

* Not all the subjects will be contacted by letter, and quite a number (e.g. foster-children and parents) will require personal contact.

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& MAZAN, J.I.

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THE BETHLEM ROYAL HOSPITAL
AND
THE MAUDSLEY HOSPITAL

DEPARTMENT OF PSYCHOLOGY
PROFESSOR H. J. BYSENCK, Ph.D., D.Sc.

INSTITUTE OF PSYCHIATRY

DE CRESPIGNY PARK

DENMARK HILL

LONDON, SE5 8AF

01-703 5411

HJE/TAM

July 6th 1972

Dr. R.C. Hockett
Acting Scientific Director
The Council for Tobacco Research - U.S.A. Inc.,
110 East 59th Street
New York 10022,
U.S.A.

Dear Dr. Hockett,

Thank you very much for your letter of July 3rd. I am happy to see that you consider the subject of my proposal to be relevant and I will be sending you the formal application when it has been approved by the Board of Studies here. I quite agree with your suggestion that it would be advantageous to separate the two questions involved, and consequently will only make an application with respect to the heritability project.

There are one or two things which I would like to say in addition to the formal application, so that you should be fully informed of the position.

1. I cannot, as yet, give the names of the people I would want to appoint to carry out the work on the project, because it is impossible to tie people down to participation in a project which has not yet been approved, and would in any case not start for six months or more. I do, of course, have several people in mind and will have no difficulties in finding suitable people when final approval has been given to the project.
2. The salaries involved are pretty realistic in terms of the here and now, but there is likely to be an increase in salaries due to the impact of our high rate of inflation and this may put out my estimates, particularly for the second and third years; increases for the first year could probably be absorbed by the budget, provided they were not too large (salary scales are negotiated for all British universities and we have no choice but to fall in line). I believe the position is quite different in the United States.

I have tried to rush the preparation of the application somewhat as I shall be going on holiday soon, and there will be no further meetings of the relevant Boards which have to approve applications before the middle of October. I would therefore be most grateful to you if you would look carefully through my application

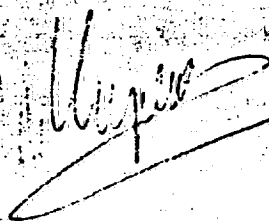
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before passing it on, and if you discover any serious deficiencies I would prefer you to send it back to me so that I could remedy these deficiencies and prepare a new submission for consideration later.

I have of course given amounts in the budget in sterling because of the uncertainties in the parity for the time being.

With many thanks for the interest you have taken in this application.

Yours sincerely,

A handwritten signature in dark ink, appearing to read 'H.J. Eysenck', with a long, sweeping horizontal line extending to the right.

H.J. Eysenck

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UNIVERSITY OF LONDON
BRITISH POSTGRADUATE MEDICAL FEDERATION



THE BETHLEM ROYAL HOSPITAL
AND
THE MAUDSLEY HOSPITAL
DEPARTMENT OF PSYCHOLOGY
PROFESSOR H. J. EYSENCK, Ph.D., D.Sc.

INSTITUTE OF PSYCHIATRY

DE CRESPIGNY PARK
DENMARK HILL
LONDON, SE5 8AF
01-703 5411

HJE/TAM

November 3rd 1972

Dr. R.C. Hockett
The Council for Tobacco Research - U.S.A. Inc.,
110 East 59th Street
New York 10022
U.S.A.

Dear Dr. Hockett,

Thank you very much for your letter of October 18th. The Board of Studies here have agreed my application and I would therefore like you to consider the application I sent in as the final one, with two minor alterations. I would like to incorporate your suggestion in your third paragraph, i.e. insert the word "determinants" in the sentence dealing with the inheritance of the smoking habit. I would also like to add the following references to the work by Lyndon Eaves, whom I was suggesting as Consultant.

EAVES, L.J. Short Communication: The multivariate analysis of certain genotype-environment interactions. Behaviour Genetics, 1972, 2, 241-244.

" The Genetic Analysis of Continuous Variation: A comparison of experimental designs applicable to human data. II. Estimation of Heritability and comparison of environmental components. Brit. J. Math. & Stat. Psychology, 1970, 23, 189-198.

" Computer simulation of sample size and experimental design in human psychogenetics. Psychol. Bull., 1972, 77, 144-152.

If you would prefer me to re-write the application I could of course do this, and if Dr. Seltzer has to make any additional suggestions, that would of course have to be done.

As regards the timing, April 1st would serve just as well as January 1st, except for one thing. We have a grant from the Medical Research Council for twin research which is running out at the end of this year, and it would be very useful to keep one or two of the people engaged on it to

Contd.....

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work on this new project, in view of their familiarity with the twin register, methodology, etc. The problem could be solved, of course, if the Council could make a special interim grant of, say, £2000 to cover the cost of the two people involved until April. I don't know if this is possible but it would certainly ease some of my problems here.

The Animal Study, for which we had a special grant from the Council, has now been completely analyzed and is being written up; I will let you have a report as soon as it is finished. The results, I am afraid, are on the whole negative; this is perhaps not surprising in view of the short duration of the grant (12 months) which meant that we had to use very strong carcinogenic agents, which may have obliterated any existing differences between the strains. I would appreciate your advice on whether it would be worth while in due course to apply for a proper grant; perhaps you could form a better judgement when you have received the report.

During a recent visit to Rome as Consultant to the Italian Ministry of Education, I made contact with some people working with various types of cancer patients and managed to inaugurate a research programme similar in some ways to what I have done with Kissen, i.e. comparing the personality of cancer patients with that of controls also coming for diagnosis. This should produce some interesting results in due course.

With kind regards,

Yours sincerely,


H.J. Eysenck

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#787A FRIEDMAN

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

December 5, 1972

3

Grant application No. 787A

To: The committee comprising Drs. Jacobson, Loosli, and Sommers

Subject: Gary D. Friedman, M.D., M.S., Kaiser Foundation Research
Institute, Oakland, Calif.

Continuation application No. 787A

"Characteristics of Smokers and Non-Smokers"

History

A two year plan was approved by the SAB in 1971:
Grant 787 was \$99,941; the current Grant 787R1 is
for \$98,980.

Application No. 787A requests \$101,100 for 1973-
1974, plus one additional year. As there is no
commitment, this competes as a new application.

Document Submitted (attached)

Application dated November 15, 1972.

Comment

The Planning Committee will be asked to consider this
application on December 8, 1972.

F.W.N.

F.W.N.

FWN:wg

Encl.

cc: Dr. Gardner

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THE COUNCIL FOR TOBACCO RESEARCH, U.S.A. INC.

110 EAST 58TH STREET
NEW YORK, N. Y. 10022

DEC 4 1972

Application For Renewal of Research Grant

First ☐ Second ☒

Date: November 15, 1972

1. Name of Investigator(s): (include title and degrees)

Gary D. Friedman, M.D., M.S., Principal Investigator
and Carl C. Seltzer, Ph.D., Co-Investigator

2. Institution &

Address: Department of Medical Methods Research
Kaiser Foundation Research Institute
3779 Piedmont Avenue
Oakland, California 94611

3. Short Title of Project:

Characteristics of Smokers and Non-Smokers

4. Proposed Renewal Starting Date: (Anniversary or other) February 1, 1973

5. Discuss any Important Changes or Additions to Objectives or Specific Aims:

Our basic objectives are unchanged. We wish to continue to provide detailed and definitive information on the characteristics of smokers and non-smokers. Our progress report describes what has been completed and what areas of work are still in progress.

There remain a number of subjects and areas of concern that have not been analysed yet. For example, much work needs to be done with our data in the area of personality characteristics, socioeconomic factors, pulmonary function tests, urine findings, morphological characteristics and medical history to name just a few subjects. In addition, we would like to begin assembling some of the data that go beyond our original objectives. This would involve looking at factors that relate to changes in smoking habits between two multiphasic examinations. Specifically, what are the characteristics of smokers who quit as compared to those that continue smoking, and what are the characteristics of non-smokers who start smoking as compared to those who remain non-smokers? We would also like to work more with some of the family history data that were not computer-stored in standard fashion, as were the other variables we have analysed.

Thus we can foresee at least two or three years of additional work along the lines described above and would hope that the Council for Tobacco Research-U.S.A. would consider a renewal of our grant on this basis,

6. Give a Brief Statement of your Working Hypothesis if altered or modified:

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7. Changes or Additions to Experimental Design and Procedures: (Attach Separate Pages)

B. Additional Requirements:

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9. Changes in Personnel with Biographical Sketches of new Personnel (append)

Dr. Loring G. Dales, a medical epidemiologist, has joined our staff and has been spending part time on this study. He supervised the final analysis and wrote the paper, dealing with serum chemistry tests, which we shall soon submit for publication. It will be highly desirable to continue his participation in this project and we have added 20% of his time to the professional personnel budget. His biographical sketch is attached.

10. Publications or Papers in Press resulting from the Project or closely related work

See progress report.

In the proposed project we will analyze data already obtained on more than 100,000 subjects who have been routinely processed in 1964-1968 through the automated multitest laboratories of the Kaiser-Permanente Medical Group, Oakland and San Francisco, California. Subscribers to the Kaiser Foundation Health Plan in the San Francisco Bay Area have had available to them a multiphasic screening survey as part of a periodic health examination. Supported by the U.S. Public Health Service and the Kaiser Foundation Research Institute, this screening survey has been developed into a standardized Automated Multiphasic Screening (AMS) procedure. (See Appendices B and C). This screening program is supervised and conducted by the same practicing physicians who furnish the medical examinations, treatment, and follow-up care.

In these laboratories, automated, electronic, and computer equipment are used as an integral part of a routine periodic health examination and an analysis of approximately 40,000 subjects annually. During the period 1964-1968, a total of approximately 170,000 examinations have been made. About two-thirds of the subjects have had more than one examination during this same period. This proposed study will deal with the data obtained on each subject's first examination regardless of how many he has taken. While the data from subsequent examinations are of considerable importance in providing information on changes in individuals over time, it is first necessary to analyze the baseline data for the initial examination. This will provide the basis for future studies of changes in the characteristics of those persons who have been examined more than once over the 1964-1968 period.

A description of the automated multitest laboratories and the procedures involved in the screening process are contained in the pamphlet entitled "The Multitest Laboratory in Health Care," and designated as Appendix D. The automated multitest laboratory provides for each subject the following data: anthropometry, an electrocardiogram, pulse and blood pressure recordings, chest x-rays, breast x-rays (women aged 48 and over), visual and hearing tests, respirometry, medical and psychological questionnaires, and laboratory tests including serum glucose, creatinine, albumin, total protein, cholesterol, uric acid, calcium and transaminase (SGOT), blood hemoglobin, white cell count, and urinalysis for pH, glucose, protein, and bacteria. (Details in Appendix E).

Anthropometric Data Provided

Height
Weight
Triceps skinfold
Subscapular skinfold
Shoulder height
Iliac crest height
Wrist height
A-P head length
A-P chest diameter
Xyphoid height
A-P abdomen diameter
A-P thigh diameter
Head breadth
Bi-deltoid diameter
Transverse chest diameter
Bi-iliac diameter
Bi-trochanteric diameter

Electrocardiogram information provided (at rest)

6 electrocardiogram (ECG) leads (AVR, AVL, AVF, V₁, V₃, V₅) are simultaneously recorded by means of a direct optical recording oscillograph. The ECGs are subsequently read by a cardiologist who records his interpretations on a "mark-sense" card. (see AMS-Electrocardiogram IBM card for various classifications).

Chest x-ray

A 70 mm posteroanterior chest x-ray is obtained, and read subsequently by a radiologist who records his interpretations on a mark-sense punch card. The x-ray is examined for abnormalities, cardiac enlargements, etc.

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Supine Pulse and Blood Pressure

Systolic blood pressure
Diastolic blood pressure
Pulse rate

Visual Tests

Visual acuity is tested by reading a wall chart, and the pupillary light reflex is tested; the results are recorded on a mark-sense card.

Visual acuity
Pupillary escape
Iris color
Ocular tension

Respirometry

FEV1
FEV2
Vital capacity, total
Peak Flow

Psychological Questionnaire

Sorting of 155 psychological questions (similar to MMPI) into "true" or "false".

Birthplace

Subject's birthplace
Father's birthplace
Mother's birthplace
Father's father
Father's mother
Mother's father
Mother's mother

OccupationDisease History of Family

Father
Mother
Father's side
Mother's side
Brothers
Sisters
Sons
Daughters

Mammography

Mammography is performed on all women aged 48 and over. Cephalocaudal and lateral views of each breast are taken. Mammograms are subsequently read by a radiologist who records his interpretation on a mark-sense card.

Hearing Tests

Hearing is tested with an automated audiometer for 6 frequencies in each ear, and the graphed readings recorded on a mark-sense card.

Medical History

Self-administered medical history questionnaire involving over 500 yes or no answers. (See Appendix E)

These questions center around symptoms, conditions and diagnoses:

Symptoms - headaches, fainting spells, pain in chest, shortness of breath, cough, vomiting, stomach pain, etc.

Conditions - urinary problems, glandular swellings, skin infections, etc.

Diagnoses - asthma, hay fever, chronic bronchitis pneumonia, TB, coronary attack, emphysema, liver disease, stomach ulcer, kidney disease, gallbladder disease, diabetes mellitus, thyroid disease, childhood diseases, etc

Past x-ray examinationsPast x-ray treatments

Occupational hazards - exposure to chemicals, x-ray, radioactivity

OperationsSerious IllnessesSmoking habitsService in Armed Forces

Weight - weight changes, maximum weight

EducationPersonal Information

Married, separated, divorced or widowed
Ages of parents' deaths
Ages of grandparents' deaths

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Laboratory Tests (many of which are derived by a multi-channel automated chemical analyzer)

Serum glucose
Cholesterol
Total Protein
Calcium
Creatinine
Albumin
Transaminase (SGOT)
Uric Acid
Hemoglobin
White cell count
Urinalysis for pH,
protein
bacteria
Blood group

Activities - number of hours spent in

sleeping or resting
self-care
leisure time
usual work
sitting
standing
exercise

The basic classification of the subjects will be derived from their smoking habits, obtained from a self-administered questionnaire. (Question numbers 335-342, 361-368 in the Interval History Questionnaire and 141-147 in the Past Medical History Questionnaire). This will permit the classification of the subjects into non-cigarette smokers, cigarette smokers by amount (less than 1 pack a day, 1-2 packs a day, 2 packs a day or more), inhalation, ex-cigarette smokers, cigarette smoking duration (less than 10 years, 10-20 years, more than 20 years), and current cigar and pipe smoking in men. The series will be further classified according to sex, age, and race (white, black, and yellow skin color). The series yields approximately 82 percent white, 12 percent black, 4 percent yellow (oriental), and 2 percent "other." Males comprise 45 percent and females 55 percent of the subjects, with a median age of 45.7 years for males and 46.0 for females (14).

The data are stored in the data processing center on computer tape. Data processing and analysis will be performed by means of an IBM 360/50 system. Generated smoking classes by age, sex, and race will be compared for morphological, physiological, socio-economic, psychological, and behavioral characteristics obtained by the AMS program. Means, standard deviation, frequency distributions, chi squares, and other tests of significance will be derived on the pertinent data.

To determine how closely the multiphasic subjects represent the general Kaiser Health Plan population, a survey will be conducted in or about October, 1970. This will involve sending questionnaires to a 5 percent (4,000) random sample of the Kaiser Health Plan population in the area served by the Oakland AMS laboratory. These questionnaires will contain the same smoking questions mentioned above, as well as other social and demographic items. Non-respondents will be contacted by an interviewer when possible.

Being a complex organism, man must not only be evaluated in a univariate, but also in a multivariate fashion. These data afford the opportunity for such analyses. But even with the large number of available variables, this is only a partial picture, since many large demographic influences must be delineated,

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such as air pollution, occupational exposures, style of life, etc. As the results of the comparisons appear, we will begin to see if there are any major groupings of characteristics which differentiate smokers from nonsmokers, and which may suggest themselves for an insight into smokers' and nonsmokers' profiles.

The nature of the study is such that its activities cannot be easily separated in 12-month periods, but should be considered as a 24-month study. However, status reports will be furnished as required, and the 12-month report will be submitted together with a projection for the next 12-month period.

Never before has such extensive and manifold data been available on such a large and varied series for analysis on the basis of smoking habits. It is the type of information that many have been seeking to help answer the question as to what extent smokers differ from nonsmokers, apart from their smoking practices. Furthermore, such data are here available for analysis without the enormous cost which would be necessary for obtaining such basic information.

1003538775

3.

11. Budget (for coming year)

CHARACTERISTICS OF SMOKERS VS NON-SMOKERS RENEWAL
FEBRUARY 1, 1973-JANUARY 31, 1974

A. Salaries (Personnel by names or category)		% time	Amount
Professional			
Gary D. Friedman, M.D., M.S.		20%	REDACTED
Carl C. Seltzer, Ph.D.		20%	REDACTED
A. B. Siegelaub, M.S.		30%	REDACTED
Loring G. Dales, M.D.		20%	REDACTED
Technical			
Programmers (2)		200%	26,400
Clerk-Typist		100%	10,440
Sub-Total			\$62,200
B. Consumable Supplies (list by categories)			
Office supplies & copying			1,100
Sub-Total			\$1,100
C. Other Expenses (itemize)			
Travel by Dr. Seltzer, Boston-Oakland (3 trips each year)			REDACTED
Travel to scientific meetings (4 trips)			20,000
Data processing			500
Key punching			
Sub-Total			\$24,300
D. Permanent Equipment (itemize)			
2 Punch card files @ \$180.00 ea.			360
Sub-Total			\$360
E. Overhead (15% of A+B+C)			
Total			\$13,140
			\$101,100

IT IS UNDERSTOOD THAT THE APPLICANT AND INSTITUTIONAL OFFICERS IN APPLYING FOR A GRANT HAVE READ AND FOUND ACCEPTABLE THE COUNCIL'S "STATEMENT OF POLICY CONTAINING CONDITIONS AND TERMS UNDER WHICH PROJECT GRANTS ARE MADE."

Signature Gary D. Friedman
DIRECTOR OF PROJECT Gary D. Friedman, M.D.

Signature Clifford H. Keene
DIRECTOR OF PROJECT Clifford H. Keene, M.D.

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4.

11. Budget (Estimated 2nd. Year)

CHARACTERISTICS OF SMOKERS VS NON-SMOKERS RENEWAL
FEBRUARY 1, 1974-JANUARY 31, 1975

A. Salaries (Personnel by names or category)	% time	Amount
Professional		
Gary D. Friedman, M.D., M.S.	20%	\$
Carl C. Seltzer, Ph.D.	20%	
A. B. Siegelaub, M.S.	30%	
Loring G. Dales, M.D.	20%	
Technical		
Programmers (2)	200%	27,850
Clerk-Typist	100%	11,020
Sub-Total		\$65,660
B. Consumable Supplies (list by categories)		
Office supplies & copying		1,100
Sub-Total		\$1,100
C. Other Expenses (itemize)		
Travel by Dr. Seltzer, Boston-Oakland (3 trips each year)		R
Travel to scientific meetings (4 trips)		R
Data processing		20,000
Keypunching		500
Sub-Total		\$24,300
D. Permanent Equipment (itemize)		
		0
Sub-Total		0
E. Overhead (15% of A+B+C)		\$13,660
Total		\$104,720

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(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME Loring G. Dales	TITLE Epidemiologist, Medical Methods Research	BIRTHDATE (Mo, Day, Yr.) R
PLACE OF BIRTH (City, State, Country) Los Angeles, Calif.	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date) U.S.A.	SEX <input checked="" type="checkbox"/> Male <input type="checkbox"/> Female
EDUCATION (Begin with baccalaureate training and include postdoctoral)		
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED
STANFORD UNIVERSITY STANFORD SCHOOL OF MEDICINE INTERNSHIP, TUFTS-NEW ENGLAND MEDICAL CENTER, BOSTON UNIVERSITY OF CALIF. SCH. OF PUBLIC HEALTH RESIDENCY, PREY. MED., U.C. SCH. OF PUBLIC HEALTH	A.B. (WITH GREAT DISTINCTION) M.D. M.P.H.	REDACTED
HONORS Phi Beta Kappa (undergraduate) R		
MAJOR RESEARCH INTEREST Epidemiology of non-infectious diseases Environmental toxicology		ROLE IN PROPOSED PROJECT
RESEARCH SUPPORT (See instructions)		
RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List 2 or most representative publications. Do not exceed 3 pages for each individual.)		
Epidemiologist, Medical Methods Research Dept., Permanente Medical Group, Oakland, California		
Assistant Research Epidemiologist, University of California School of Public Health, 1970-71.		
Project examination coordinator, Japanese American Health Research Program, 1969.		
Medical Officer, U.S. Public Health Service Indian Hospital, Rapid City, South Dakota, 1966-1968.		

IHS JDB
Rev. 3-70

1003538778

PUBLICATIONS (Dr. Loring Dales)

1. Dales L., Kahn, E., Wei, E.: Methylmercury poisoning. Calif. Med. 114: 13-15, 1971.
2. Dales, L.G. : The neurotoxicity of Alkyl Mercury Compounds. Am. J. Med. 53: 219-232, 1972.

1003538779

Department of Medical Methods Research
Kaiser Foundation Research Institute
3779 Piedmont Avenue
Oakland, California 94611

October 15, 1971-November 15, 1972

CHARACTERISTICS OF SMOKERS AND NON-SMOKERS

This report summarizes our activities since our first progress report of October 15, 1971.

Our initial retrievals and tabulations of all multiphasic variables for smokers and non-smokers are essentially complete. In carrying these out, we focussed on a population of about 89,000 subjects who had multiphasic examinations between 1964 and 1968, and who were classified as having white, black or yellow skin color, and who gave consistent and complete enough answers to questions about smoking that they could be classified with confidence into one of the smoking categories that we defined.

We have generated many volumes of computer output similar in format to those shown as Appendix B in our first progress report. Our efforts of late have been directed mostly towards looking at specific subject matter areas in depth, in order to present and publish our findings in a way that will be meaningful to the scientific and medical community. We have made much progress toward this goal as will be outlined below, but there is still a great deal of work to do in the future if we are to take full advantage of the Kaiser-Permanente data base in characterizing smokers and non-smokers.

The various studies that have been fully or partly completed to date are summarized below. Results that have not yet been published are presented as confidential privileged information.

Smoking Among White, Black and Yellow Men and Women:
Kaiser-Permanente Multiphasic Health Examination Data, 1964-1968.

This basic paper provides a description of our study population and

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describes our methods for assessing and classifying smoking habits. Detailed data about smoking habits are presented according to age, sex and race breakdowns. To our knowledge, this is the first large scale U.S. study to provide information on the smoking patterns in "non-whites", subdivided into blacks and orientals.

This has been published in the American Journal of Epidemiology (96:23-35, 1972) and 20 reprints have been provided to the Council for Tobacco Research-U.S.A.

Smoking Habits and the Leukocyte Count:

This study explores in detail the relationship between smoking habits and the leukocyte count, a relationship which has been discussed very little in the literature. In our data cigarette smokers had, on the average, substantially higher leukocyte counts than non-smokers, with exsmokers and cigar and pipe smokers being intermediate. The leukocyte count was related to quantity smoked, inhalation and smoking duration. We looked at a number of variables to try to explain the smoking-leukocyte count relationship. The presence of chronic bronchitis explained a small part of the difference, but for the most part, the smoker-nonsmoker difference in leukocyte counts could not be attributed to disease or to extraneous factors. Most of the changes in leukocyte counts in persons who started and stopped smoking were consistent with an effect of smoking.

This study was presented to the Society for Epidemiologic Research, Houston, Texas, May 6, 1972. It has been accepted for publication in the Archives of Environmental Health, and is scheduled to appear in the February or March, 1973 issue.

Smoking and Drug Consumption in White, Black and Oriental Men and Women:

This study fills an important gap in knowledge about the drug usage characteristics of smokers and non-smokers. While some information has been published about drug addiction and oral contraceptive usage in smokers and

non-smokers, little if any information is available about differences in usage of the major classes of medicinal drugs. Our analysis of questionnaire responses of smokers and non-smokers has revealed some striking differences in the percentage who take various drugs, particularly in the younger age groups.

This paper is complete and has been submitted to Clinical Pharmacology and Therapeutics.

Cigarette Smoking Habits and Serum Chemistry Tests:

Our data analysis with regard to differences between cigarette smokers in serum chemistry tests has revealed some striking and interesting differences. On the average, creatinine and albumin levels were lower in the smokers of both sexes, while the opposite was true for one-hour post-challenge serum glucose. Globulin levels were consistently lower in the women smokers only. Uric acid concentrations were lower in the men who smoked. Cholesterol levels were higher in the white men who smoked but not in the black male smokers. Calcium and SGOT levels of smokers were quite similar to those of non-smokers.

Where the serum chemistry concentrations of the smokers differed from those of non-smokers, the associations were examined for relation to 1) the interaction of other variables correlated with smoking and 2) the quantity of cigarettes smoked. In the cases of serum albumin, uric acid, and cholesterol the evidence, while not constituting proof, was consistent with the hypothesis that cigarette smoking directly caused the difference. The difference noted between smokers and non-smokers in serum glucose concentration appeared to be partially related to differences in alcohol consumption habits. In particular, it was of great interest to note that among non-drinkers, smokers did not have high serum glucose levels.

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A manuscript has been completed and is now undergoing some final editing and minor revisions. We plan to submit it to the Annals of Internal Medicine.

Cigarette Smoking and Exposure to Environmental Hazards:

In analyzing the multiphasic questionnaire data we have noted that more cigarette smokers reported occupational exposure to environmental hazards than did non-smokers. These hazards included chemicals, gases, engine exhaust fumes, metal and plastic fumes, various dusts, extreme heat and loud noise. Since these exposures have important pathogenetic effects they should be taken into account in population studies of smoking and health. Accordingly, we plan to publish these findings. A first draft of a manuscript has been completed except for one section: we are presently interviewing a sample of multiphasic screenees to validate the questionnaire responses regarding these exposures, and to gain more insight into exactly what is involved when a person reports, for example, an exposure to loud noise.

Smoking, Hearing Loss, and Exposure to Loud Noise:

The National Health Survey found an increased prevalence of hearing loss in male heavy smokers. We also found a greater prevalence of hearing loss in smokers than in non-smokers. However, because we also noted increased exposure to loud noise among smokers (see above) it seemed important to take noise exposure into account in analyzing differences in hearing loss between smokers and non-smokers. These analyses have been completed. We have found that, compared to the much stronger relationship of hearing loss to age, sex and noise exposure, smoker-nonsmokers differences are relatively slight.

A first draft of this manuscript is mostly completed and requires only some additional review of the literature.

Smoking and Response to Health Questionnaires:

During the first year, aided by CTR support, Dr. Thomas Oakes conducted a survey of a five percent random sample of Kaiser Health Plan Members in the Oakland-Berkeley area. Some results of this survey were mentioned in our

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first published paper. In addition, other pertinent studies have been made with these data. Responses to the survey questionnaire came in by successive waves, each requiring an additional letter or other means of persuasion to gain cooperation. Thus it was possible to grade or categorize respondents according to their degree of cooperativeness in completing a health and social questionnaire. In correlating these with their smoking habits we have noted that, on the average, smokers respond less readily and promptly than non-smokers. The most cooperative group were the exsmokers. These findings suggest that there are important psychological differences between smokers, exsmokers, and non-smokers. The analysis also involves looking at cooperativeness in terms of disease status.

A first draft of this manuscript is complete and is undergoing some final editorial revisions.

Smoking and the Use of Medical Care Facilities:

Based on the same survey Dr. Oakes is completing a manuscript about the relationship of smoking to the use of medical care facilities including multiphasic checkups, other checkups, doctors' office visits and hospitalizations.

Several differences were noted between smokers and non-smokers in the use of these medical care services. For example, smokers took fewer checkups than non-smokers.

A first draft of this manuscript is complete and is undergoing some final editorial revisions.

Multivariate analyses to discriminate between smokers and non-smokers:

Dr. Ury has been analysing groups of questionnaire responses in an effort to find those combinations that discriminate best between smokers and non-smokers.

Work so far has focussed on the neuromental or psychological questions which, individually were the best discriminators. The methods tried so far have not proven to be very effective but others are currently being tested.

This work is important from both a methodological and subject-matter

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point of view. It would be of value to identify those combinations of characteristics that best characterize smokers. Also it will be helpful to other research workers in this area to learn which statistical methods are the most powerful.

Distinctions between Cigar and Pipe Smokers

Most studies of smoking effects have grouped cigar and pipe smokers into one category. Dr. Seltzer has recently published findings from Veterans Administration data showing some striking differences between cigar and pipe smokers regarding both body build and socioeconomic characteristics.

A detailed comparison of the characteristics of cigar and pipe smokers is now in progress using our data. This will provide a much more definitive study of the question since larger numbers of subjects are involved.

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#58D BHAGAT

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PHARMACOLOGY

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THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

November 28, 1972

3

Grant application No. 588D

To: The committee comprising Drs. Bing, Cattell, and Jacobson

Subject: B. Bhagat, Ph.D., St. Louis University School of Medicine,
St. Louis, Missouri
Continuation application No. 588D
"Effect of Chronic Administration of Nicotine and Smoking
on Brain Biogenic Amines"

History

Dr. Bhagat has been supported through Grant No. 588 with renewals, continuations, and supplements, since 1967. An earlier application was denied but for reasons of program balance, not on scientific merit.

Studies supported by CTR to date have been productive of publications.

Application No. 588D requests \$36,805, plus two additional years.

Document Submitted (attached)

Application dated November 17, 1972.

Comment

The applicant proposes to add tobacco smoke exposure to the treatments he has studied to date. On September 22, 1972 he visited the CTR office for discussions with staff.

As a January 1, 1973 starting date is requested, the Planning Committee will be asked to take interim action on December 8.

F.W.N.
F.W.N.

FWN:wg
Encl.

cc: Planning Committee

1003538788

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 50TH STREET
NEW YORK, N. Y. 10022

Application For Research Grant

NOV 27 1972

Date: November 17, 1972

1. Name of Investigator(s): (include Title and Degrees)

B. Bhagat, Ph.D.
Professor of Physiology

2. Institution &

Address:

Department of Physiology
St. Louis University School of Medicine
1402 South Grand Boulevard
St. Louis, Missouri 63104

3. Short Title of Project:

Effect of Chronic Administration of Nicotine
and Smoking on Brain Biogenic Amines

4. Proposed Starting Date:

January 1, 1973

5. Anticipated Duration of this Specific Study:

3 years

6. Brief Description of Objectives or Specific Aims:

See Proposal.

7. Give a Brief Statement of your Working Hypothesis:

See Proposal.

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8. Details of Experimental Design and Procedures: (Attach Separate Pages)

See Proposal.

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

See Page 2A.

10. Additional Requirements:

None.

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11. Biographical sketches of all principal and professional personnel (append)

See pages 2C through 2G

12. List of publications: (Five most recent as pertinent) (append)

See page 2R.

FACILITIES AVAILABLE

1. Space. Our laboratory and office (approximately 600 sq. ft.) is well equipped with all the standard facilities. In addition, a cold room, radio-isotope room, animal operating room, and machine shop are also available.
2. Equipment. In addition to standard laboratory equipment, such as glassware and other apparatus, the following items are available for our use: Grass stimulator, spectrofluorometer (Aminco-Bowman), mechanical shaker, and International portable refrigerated centrifuge.
3. Animal Research Space. Ample animal and laboratory space is available in the new renovated Animal Care Facility at St. Louis University Medical Center to permit proper conduct of these studies. These quarters are under the direction of a veterinarian who quarantines and conditions animals prior to use in experiments.
4. Library. An excellent medical library supports the research service. It includes over 5,000 volumes and regularly subscribes to 189 scientific periodicals. An excellent interlibrary loan system with the four Universities and two medical societies in our area gives us ready reference material promptly. The Yalem Computer Center of St. Louis University is readily available for the processing of data and gives a priority to medical research.

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12. List of publications: (Five most recent as pertinent)

1. B. Bhagat: Effect of chronic administration of nicotine on storage and synthesis of noradrenaline in rat brain. Br. J. Pharmac. 38: 86, 1970.
2. B. Bhagat: Influence of chronic administration of nicotine on the turnover and metabolism of noradrenaline in the rat brain. Psychopharmacologia (Berl.) 18: 325, 1970.
3. B. Bhagat and M.W. Rana: Effect of chronic administration of nicotine on the concentrations of adrenal enzymes involved in the synthesis and metabolism of adrenaline. Br. J. Pharmac. 43: 250, 1971.
4. B. Bhagat, T. Bayer and C. Lind: Effect of chronic administration of nicotine on drug induced hypnosis in mice. Psychopharmacologia (Berl.) 21: 287, 1971.
5. P. Chang, B. Bhagat and J.C. Taylor: Effect of chronic administration of nicotine on acetylcholinesterase activity in the hypothalamus and medulla of the rat brain. An Ultrastructural Study. Brain Res. (In press).

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Page 2C.

CURRICULUM VITAE

BUDH DEV BHAGAT, Ph.D.

Born:

R

Education:

Ph.D., Pharmacology, (Faculty Medicine),
London University, R

Postdoctoral in Pharmacology,
University of Wisconsin Medical School, R

Postdoctoral in Pharmacology,
University of Minnesota Medical School, R

Faculty Appointments:

Assistant Professor, Department of Pharmacology,
Howard University Medical School, 1964-66

Assistant Professor, Department of Pharmacology,
New York Medical College, 1966-68

Associate Professor, Department of Physiology
Associate Professor, Department of Pharmacology,
St. Louis University School of Medicine, 1968-71

Professor, Department of Physiology,
Professor, Department of Pharmacology,
St. Louis University School of Medicine, 1971-

Major Research Interests:

Autonomic Nervous System, Neurotransmitter, Cardiovascular

Committee Appointment:

Member - Advisory Board for "Neurosciences Research", Academic Press

Publications:

Approximately 169 publications to date.

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Page 2D.

CURRICULUM VITAE

CHANDRA HANS MISRA, Ph.D.

Born:

R

Education:

B.Sc., Physics
Lucknow University, R

M.Sc., Chemistry
Lucknow University, R

Ph.D., Chemistry
Lucknow University, R

Experience and Appointments:

Research Associate, Department of Physiology
St. Louis University School of Medicine, 1972-

Postdoctoral Fellow, Department of Biochemistry
St. Louis University School of Medicine, 1971-1972

Research Associate, Endocrinology Research
Veterans Administration Hospital, St. Louis, Mo. 1970-1971

Assistant Research Officer, Department of Pharmacology
K.G. Medical College, Lucknow, 1969-1970

Research Assistant, Department of Pharmacology
K.G. Medical College, Lucknow, 1965-1969

Professional Organizations:

President of Chemical Association,
Lucknow University, 1961-62

Member of Association of Physiologist and Pharmacologist of India,
1965-66

Member of Indian Pharmacological Society, 1968-

Member of International Society for Biochemical Pharmacology

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Research Publications:

1. STUDIES ON COMPLEX COMPOUNDS II. Co-ordination Compounds of Mercury and Aromatic Amines. C.H. Misra, S.S. Parmar and S.N. Shukla, J. Inorg. Nucl. Chem. 28, 147 (1966).
2. STUDIES ON COMPLEX COMPOUNDS II. Co-ordination Compounds of Mercury and Disubstituted Anilines. C.H. Misra, S.S. Parmar and S.N. Shukla, J. Inorg. Nucl. Chem. 29, 2589, (1967).
3. STUDIES ON COMPLEX COMPOUNDS III. Copper and Mercury Complexes of 2-chloro-6-methylaniline and 5-chloro-2-methylaniline. C.H. Misra, S.S. Parmar and S.N. Shukla, Can. J. Chem. 45, 2459 (1967).
4. STUDIES ON COMPLEX COMPOUNDS IV. Copper Complex of Quinazolone Hydrozides. C.H. Misra, S.S. Parmar and R.C. Arora, Inorg. Nucl. Chem. Letters 3, 603 (1967).
5. STUDIES ON COMPLEX COMPOUNDS V. Co-ordination Compounds of Mercury and Pharmacologically Active Amines. C.H. Misra, S.S. Parmar and S.N. Shukla, Can. J. Chem. 46, 2485 (1968).
6. STUDIES ON COMPLEX COMPOUNDS VI. Copper Complexes with Pharmacologically Active Cyclohexylamine Derivatives. C.H. Misra, S.S. Parmar and J.P. Barthwal, Can. J. Chem. 47, 4705 (1969).
7. STUDIES ON COMPLEX COMPOUNDS VII. Copper Complexes of Thiosemicarbazones as Possible Anticancer and Antitubercular Compounds (Communicated). (In Press).
8. STUDIES ON COMPLEX COMPOUNDS VIII. Metal Complexes of Schiff's Bases as Antibacterial (Communicated). (In Press).
9. Evidence Towards Possible Stimulation of Catecholamine Biosynthesis by Copper-Tyrosine Complex. S.S. Parmar, J.P. Barthwal, K.P. Bhargava, and C.H. Misra. Fifth International Congress on Pharmacology, San Francisco.
10. Studies on the Activity of Enzymes in Thyroglobulin and Albumin Induced Immunity at Different Tissue Levels. (In Preparation).
11. Analytical Application of the Schiff's Base for Iron Estimation. (In Press).
12. STUDIES ON COMPLEX COMPOUNDS IX. Metal Chelates of Bigunides. (In Preparation).
13. Antibacterial and Antifungal Properties of Some New Mercury Complexes. (In Press).

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CURRICULUM VITAE

YU-CHIANG LEE, Ph.D.

Born:

R

Education:

B.S., Chemistry, Taiwan Christian College, R

Ph.D., Biochemistry, Oklahoma State University, R

Experience and Appointments:Research Associate, Department of Physiology
St. Louis University School of Medicine, 1971-Postdoctoral Fellow, Department of Biochemistry
The University of Texas Medical School at Dallas, 1969-1970Research Assistant and Graduate Student, Department of Biochemistry
Oklahoma State University, 1964-1969Laboratory Instructor, Department of Chemistry
Taiwan Christian College, 1960-1963Professional Organizations:

Member,

REDACTED

Member,

Member,

R

Publications:

1. K. Yamaguchi, Y.C. Lee, and R.K. Gholson. Nicotinamide methyl transferase on the regulation of NAD biosynthesis. International Congress of Biochemistry IV-F-165 (1967).
2. Y.C. Lee, R.K. Gholson and Nicholas Racia. Identification of Two New Nicotinamide Metabolites in Germ Free Rats. A.C.S. National Meeting (1968) Abstract Biol. 24.
3. Y.C. Lee, R.K. Gholson and Nicholas Racia. Isolation and Identification of Two New Nicotinamide Metabolites. J. Biol. Chem. 244, 3277 (1969).

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4. Y.C. Lee, R.M. McKenzie, R.K. Gholson and Nicholas Racia. A Comparative Study of the Metabolism of Nicotinamide and Nicotinic Acid in Normal and Germ Free Rats. Biochem. et Biophys. Acta 264, 59 (1972).
5. Taruna Dave, Y.C. Lee, R.J. Bryan, R.C. Srivastava and B. Bhagat. Activity of Enzyme Involved in Synthesis and Degradation of Norepinephrine in Guinea Pig Isolated Vas-Deferens During Intermittant Nerve Stimulation. Federation Abstracts 31, 543, 1972.
6. B. Bhagat, T. Dave, Y.C. Lee and R.J. Bryan. Nerve Stimulation and the Activity of Catecholamine Metabolizing Enzyme in Guinea Pig Isolated Vas-Deferens. Amer. J. Physiol. (in press)

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13. Budget (1st year)

A. Salaries (Personnel by names)

Professional

B. Bhagat, Ph.D.
C. H. Misra, Ph.D.
Y.-C. Lee, Ph.D.

% time

20%
100%
100%

Amount

REDACTED

Technical

R. J. Bryan
Animal Caretaker

100%
50%

REDACTED

Fringe Benefits

Sub-Total

B. Consumable Supplies (list by categories)

Radioactive material
Drugs and chemicals
Animals
Maintenance of equipment

1,000.
1,000.
2,500.
400.

Sub-Total

4,900.

C. Other Expenses (itemize)

Travel to attend National meetings
Photographic material
Reprint costs

400.
200.
200.

Sub-Total

800.

D. Permanent Equipment (itemize)

NONE

E. Overhead (15% of A+B+C)
(exclusive of fringe benefits)

4,605.

Total

\$36,805.

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	REDACTED	4,900.	800.	--	4,605.	36,805.
Year 3	REDACTED	4,900.	800.	--	4,605.	36,805.

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature

Director of Project

(314) 865-2288 x. 412

Telephone

Signature

Business Officer of the Institution

Telephone

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Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current

Title of Project	Source	Amount	Duration
Nicotine Behavioral Changes and Brain Biogenic Amines	National Institute of Mental Health	\$24,947.	7/1/72 - 6/30/73

Pending

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INTRODUCTION

Ever since smoking became established, it has enjoyed such striking success that there can be little doubt that the habit is rewarding to the participant. And since the discovery, more than a century ago, that the most powerful pharmacological agent in tobacco is nicotine, the obvious view has often been expressed that people who use tobacco are seeking the physiological responses to this alkaloid. Johnston (Lancet, 11:792, 1942) reported that the injection of nicotine resulted in a pleasant sensation to smokers, whereas nonsmokers reported an unpleasant sensation. And Deneau and Inoki (Ann. N.Y. Acad. Sci. 142:277-279, 1967) found that monkeys may even administer nicotine intravenously to themselves. These findings strongly suggest that nicotine is the active constituent of tobacco smoke and that the effects of nicotine from smoking can be imitated by intravenous injection of this alkaloid.

Recently Goldfarb et al (Psychopharmacologica 17:89, 1970) compared subjects' base line smoking rate with their own brands of cigarettes to their rates when smoking specially prepared lettuce cigarettes varying in nicotine content from zero to 2-25 mg nicotine per cigarette. They found that subjects do perceive differences in nicotine content of cigarettes. Armitage et al (Nature, 217, No. 5126:313, 1968) showed that small amounts of nicotine injected intravenously usually increased the lever pressing activity of rats and caused a change in the EEG of cats, indicative of cortical activation. These results in experimental animals are consistent with the subjective impressions of some smokers that inhalation of tobacco smoke causes them to be more alert and efficient. Thus, their findings suggests one good reason for the extraordinary fact that despite the widely publicized risks, many billions of cigarettes are used in this country alone every year. It appears that nicotine produces highly describable effects upon the brain. It seems likely that some people smoke in order to dose themselves with nicotine.

Drugs which produce behavioral changes in man and animals can also produce changes in brain catecholamine patterns. Neurochemical, pharmacological and behavioral studies have provided a number of findings compatible with this hypothesis. Reserpine, a hypotensive and tranquilizing agent which characteristically depletes the brain of norepinephrine and other amines, has been observed in a significant proportion of patients receiving it to result in a state closely resembling endogenous depression. On the other hand, a number of drugs which elevate mood and have been found of value in the treatment of depression appear to act on central norepinephrine in ways which could increase its physiologically active concentration, either by inhibiting the enzyme responsible for its presynaptic deamination, by favoring its release or by inhibiting its reuptake, presumably at the central synapse (Kety et al, Proc. N.A.S. 58:1249, 1967).

Recently, tricyclic antidepressant drugs have been shown to increase the rate of synthesis of brain norepinephrine (Neff and Costa, 1967). The therapeutic efficacy of electroconvulsive shock in depression had been explained in its ability to induce acceleration in the synthesis of norepinephrine. Ryo Takahashi et al (1968), presented evidence that indicates that overproduction

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of catecholamines might occur in the patient during the manic state and underproduction during the depressive state.

The catecholamines (norepinephrine, epinephrine and dopamine) and indoleamine (serotonin) are the most important brain amines. Norepinephrine is present in various regions of the mammalian brain, particularly in the hypothalamus. Epinephrine also occurs in the brain in high concentrations in that area. Highest concentrations of dopamine are found in the basal ganglia; lower concentrations of this amine are found in most other brain areas. Serotonin is present in appreciable amounts in the brain and its distribution is similar to that of epinephrine and norepinephrine.

It has been suggested that nicotine might produce some of its actions in the central nervous system by the release of norepinephrine or other amines from stores in central nervous system tissue (Vogt, Proc. 4th Int. Cong. Biochem., F. Brucker, Ed., 3:279). Several workers (Hansson et al, Arch. int. Pharmacodyn. 1964:148, 153; Westfall and Watts, J. Neurochem. 1964: 11, 397) measured catecholamines in the brain after single or repeated injections of nicotine, but failed to observe any clear-cut changes in the norepinephrine content, although there was some indication of change in serotonin content. Recently, Westfall et al (Ann. N.Y. Acad. Sci. 1967: 142, 83) determined the effect of nicotine on subcellular distribution of catecholamines, and showed that no significant effect on the N.E. content was observed after 0.5 mg and 1 mg/kg nicotine i.p. in whole mouse brain, and of rat diencephalon while a significant decrease in dopamine content of the brain was observed. These observations did not give clear conclusive results. The next logical step was taken in this laboratory where, with the help of labelled norepinephrine, it was shown (Bhagat, B., Kramer, S.Z., and Seifter, J., Europ. J. Pharmac. 2:234-235, 1967) that even very small doses of nicotine caused an increased release of ^3H -norepinephrine from the brain. Assuming that the release of ^3H -norepinephrine reflects an accompanying released endogenous norepinephrine then the failure to find a decrease in the concentration of cerebral norepinephrine must, in turn, reflect a rate of replenishment equal to the rate of release.

Later, in 1970 in this laboratory, the effect of chronic administration of nicotine on catecholamine concentration in the rat brain was determined (Bhagat, Br. J. Pharmac. 38:86-92, 1970). Nicotine was administered to rats 5 times a day for 6 weeks. When calculated in terms of body weight this is equivalent to 3 packs of cigarettes a day. It was found that following the chronic administration of nicotine the endogenous levels of neurohormones in the brain were unaltered, but that there was a clear increase in both the synthesis and the utilization of these neurohormones. These results were true for norepinephrine, dopamine, acetylcholine and 5-hydroxy-tryptamine. The important conclusion was obvious. Endogenous levels of neurohormones in brain tissue are deceptive indicators of the responses of the tissues to stress; instead, the important factor in the response of neurohormones to nicotine is their rate of turnover which provides a yardstick of central nervous sympathetic activity. These observations suggest that chronic administration of nicotine causes adaptive changes in the body so that there is a sustained rise in the rate of synthesis of norepinephrine to meet the requirement for norepinephrine in the face of continued enhanced utilization.

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This may also help to explain the increase in the lever pressing activity of rats given nicotine frequently (Armitage et al, 1968) and the improvement in the learning ability of rats and mice in several different tests after low subcutaneous doses of nicotine (Bovet et al, 1967).

Yet, while the rates of synthesis and utilization were increased by repeated administration of nicotine, endogenous levels continued to remain stable. It is suggested that alterations in the turnover rates rather than levels may be a correlated of nicotine induced behavioral changes. Norepinephrine is in a dynamic state of release, metabolism and biosynthesis, yet despite these changes the absolute levels of tissue norepinephrine remains remarkably constant. In order to understand the effect of smoking or chronic administration of nicotine on the catecholamine pattern and to obtain more meaningful data it is essential to study all the aspects of synthesis, storage, disposition and metabolism of catecholamine.

Only then can we obtain useful information which might alter or enhance our knowledge about the effects of smoking on the central nervous system.

ATMS

In our proposed study, animals will be exposed to tobacco smoke (or simulated atmosphere) under conditions comparable to those of human smoke exposure. Other animals will be treated with nicotine or cotinine, a metabolite of nicotine. We will examine brain and cardiovascular tissue for changes in the pattern of catecholamine, 5-hydroxytryptamine, and acetylcholine for the following reasons:

1. a) To determine the manner in which smoking affects the brain particularly its chemistry.
b) To determine whether the changes in the chemistry of brain induced by smoking are identical to that caused by nicotine.
c) To determine whether nicotine-induced alterations are due to nicotine per se or mediated through its metabolite cotinine.
2. To develop a more detailed understanding of short and long term time-courses in the altered rate of synthesis and utilization of neurohormones in the central nervous system and cardiovascular tissues. We will examine animals at specific times following the start of smoking or chronic treatment with nicotine or cotinine and once the maximum changes have developed, during the subsequent period of withdrawal of the drug. An understanding of these factors is essential to attempt to define the mechanism involved in the synthesis and metabolism of these neurohormones.
3. To examine the effect of smoking, nicotine or cotinine on certain behavior and to correlate the changes in acquired behavior with changes in the pattern of neurohormones.

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4. To try to define the mechanism involved in the changes in the pattern of neurohormones after smoking or nicotine by modifying or preventing those changes by pharmacological agents,

EXPERIMENTAL PROCEDURE

1. Preparation of Animals

Rats (Holtzman strain) weighing about 60 to 70 gm will be used throughout this study. Animals will be placed in cages which will be kept under similar conditions of lighting and humidity in a room maintained at a temperature within the range of $21.0 \pm 0.5^{\circ}\text{C}$. Food and water will be supplied ad libitum. No more than 6 rats (unless otherwise required) will be housed in each cage, since it was observed that crowding of animals increased the tyrosine hydroxylase by 32%. All animals will be acclimatized to the new environment for a period of one week before they are subjected to any treatment.

2. Body weight

Body weight will be recorded weekly.

3. Food and Water

Food and water intake will be measured daily and expressed per 100 gm of body weight.

4. Measurement of Systolic Blood Pressure

The systolic blood pressure will be measured weekly in unanesthetized animals using a pulse transducer applied to the tail.

5. Sex Difference

Whether there is a sex difference in the effect of smoking or nicotine, experiments in the females will be compared with males. Some experiments will be performed on pregnant rats.

6. Chronic Treatment of Nicotine or Cotinine

Nicotine or cotinine will be injected in various doses ranging from 0.05 to 1 mg/kg. Each dose will be injected subcutaneously, 3 times a day for at least 10 weeks. Control animals will be injected with equivalent amounts of saline. Animals will be used for study 12 hours after the last injection of nicotine.

7. Exposure to Smoke

Animals will be conditioned for at least one week prior to smoke exposure. Rats will be inserted into the animal cone holder and placed on the operating

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machine without cigarettes, three times each day for 10 minutes. Suitably conditioned animals will enter the cone holders voluntarily.

Animals losing weight generally more than one gram per day during the conditioning period will be discarded, since these animals will not survive a chronic exposure. Following one week's exposure without smoke rats will be adapted with smoke to cigarette-concentration smoke for 8 minute exposure, 3 times a day.

The Walton Horizon Smoke Exposure Machine (developed under contract by the Council for Tobacco Research, U.S.A.) will be used. It has a capacity to expose 12 young rats to tobacco smoke (or simulated atmosphere) under conditions comparable to those of human smoke exposure.

Essentially smoke will be produced by "positive" puffing (blowing) metered air through a horizontally held cigarette enclosing in a plastic dome during a timed two second puff. The two second puff interval is defined as the interval when the dome is in contact with the cigarette holder plate. The average puff volume is defined as the average puff volume of smoke produced during the first eight puffs. The 35 ml is the average puff volume of smoke produced during the first eight puffs.

In the normal one minute cycle of operation the two second puff will be followed by a 15 sec hold period, i.e., for a total exposure time of 17 sec. This will be followed by a thirty sec. purge period to sweep out the smoke and a 13 sec rest period. The smoke will be pushed into a constant volume (384 cc) smoke exposure chamber. Uniform mixing will be achieved with a mechanical mixer attached to one of the animal cone holder plates.

Animals (conditioned for at least one week prior to smoke exposure) will be held in cone shaped holders and will breathe the exposure chamber contents with their noses just inside the smoke chamber. They will be removed from the cone holder promptly after exposure to avoid water loss due to sweating and the additional stress of excessive confinement.

Cigarettes

Kentucky reference cigarettes (IRI) with different levels of nicotine will be used. They will be equilibrated for at least 24 hr at 76 (+2)°F to to (+2)% relative humidity atmosphere, by placing them unwrapped, with package opened into a dissipator (on wire mesh shelves) containing a 74% w/w glycerol-water solution in the bottom compartment. The cigarettes will be placed loosely into the chamber.

8. Behavioral Studies

A) Spontaneous motor activity. It will be measured for 15 min periods in doughnut-shaped cages 12 inches in diameter with 3 inch wide circular runways. Four equally-spaced 1 inch panels in the floor of each cage activate microswitches connected to an electromechanical counter. Measurement will be conducted in a ventilated, sound-insulated box containing four activity cages.

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For consecutive daily measurements, animals will be always placed in the same cage. Before nicotine treatment will begin, all rats will be subjected to six or seven daily 15 min sessions during which they will develop an accommodation.

B) Rotarod Performance will be measured by placing the rats on a cylinder about 5 inches in diameter and 6 inches long, which will be rotated eight revolutions per min by an electric motor. Those animals that learned to walk the rod for a period of at least 3 minutes will be given treatment.

C) Conditioned avoidance behavior will be examined by a shuttle-box technique (Rech, J. Pharmacol. exp. Ther. 146: 369, 1964). Each avoidance trial will be initiated by the conditioned stimulus, a small light activated on the side of the cage occupied by the rat. After 5 seconds the grid floor on that side of the cage will be electrified for an additional 5 sec, after which both the light and shock will be terminated together. If the rat moved to the unlighted side during the initial 5 sec, the response is scored as an avoidance. The trial will be repeated every 30 sec and 20 inch trials constituted a test session. Only rats which will average more than 15 out of 20 avoidance responses after training will be used in this study and will be given nicotine treatment.

9. Catecholamines

All aspects of catecholamine pattern (synthesis, storage, release, uptake, disposition and catecholamine enzyme - tyrosine hydroxylase (TH), monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT)) will be determined.

a) Brain

The metabolism of central monoamines is studied after labeling of central aminergic neurons by injection of microquantities of radioactive amines or their precursor in the cerebrospinal fluid. Changes in synthesis or utilization of radioactive amines can be estimated by measuring the activity of the amines in the various structures of the brain as a function of the time. In vivo release of monoamines can be estimated by superfusing a local area of the brain rich in aminergic terminals and thus by collecting radioactive amines previously synthesized from their labelled precursor.

The brain of animals at various intervals of treatment will be studied for the following parameters: 1) endogenous norepinephrine, 2) its capacity to uptake and accumulate ^3H -norepinephrine, 3) the rate of metabolism of ^3H -norepinephrine, 4) the rate of conversion of ^3H -tyrosine to ^3H -norepinephrine, 5) monoamine oxidase activity, 6) catechol-o-methyl transferase activity, 7) tyrosine hydroxylase activity, 8) disposition of dopamine, 9) rate of conversion of ^3H -choline to ^3H -acetylcholine, and 10) rate of conversion of ^{14}C -tryptophan to ^{14}C -5-HT. In order to gain more information, the brain will be divided into various parts (telencephalon, hypothalamus, cerebellum, pineal body, colliculi, pons, medulla) and in each of these parts all the parameters will be measured.

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b) Cardiovascular tissue

Norepinephrine in the tissue innervated with sympathetic nerve endings is inactivated by at least three mechanisms: a) uptake and storage in nerve terminals, b) o-methylation by catechol-o-methyl transferase (COMT) and c) oxidative deamination by monoamine amine oxidase (MAO). Inactivation by uptake of norepinephrine is more important than inactivation by metabolism. In support of this is the observation that physiological effects of injected norepinephrine are rapidly terminated, even after both MAO and COMT are inhibited.

In the heart and other sympathetically innervated organs, adrenergic nerve terminals are distributed throughout the entire tissue, whereas in the blood vessels, adrenergic nerve terminals are confined only to the adventitia and the underlying portions of the media. For this reason, catecholamines are inactivated differently in the vascular smooth muscle. In the adventitia and underlying portion of the media, like any other organ, the inactivation by binding seems to predominate over inactivation by enzymatic destruction, whereas in the greater part of the media norepinephrine is primarily activated not by uptake but by enzymatic breakdown. Cocaine, which is known to block the uptake of norepinephrine and thereby causes supersensitivity of the organ to norepinephrine, confines its potentiating action to the adventitia only, but does not affect the uptake of norepinephrine in the media.

Recently Berkowitz et al (J. Pharmac. exp. Ther. 177:119, 1971) have shown an uneven regional distribution in the blood vessels: the distal portions of the aorta and mesenteric artery had twice the content of the proximal tissue. Blood vessels take up less than 20% as much norepinephrine ^3H from the circulation as the heart.

Since smoking is implicated in the development of cardiovascular diseases and particularly to death from coronary heart disease, it is therefore necessary to determine the effect of smoking or chronic administration of nicotine on the synthesis and disposition of norepinephrine in the cardiovascular tissues. The following tissues will be examined: adrenal gland, superior cervical ganglia, heart, aorta, superior mesenteric artery, renal arteries, abdominal (inferior) vena cava and mesenteric vein. Changes in catecholamine pattern will be determined at various intervals following treatment and following withdrawal. The following parameters will be measured in the adrenal gland: 1) norepinephrine, 2) epinephrine, 3) TH activity, 4) MAO activity, 5) COMT activity, 6) phenyl-ethanol-N-methyl transferase.

The following parameters will be measured in superior cervical ganglia: 1) norepinephrine, 2) TH activity, 3) MAO activity, 4) COMT activity.

The following parameters will be measured in heart and vascular tissues: 1) norepinephrine, 2) capacity to take up and accumulate ^3H -norepinephrine, 3) the rate of metabolism of ^3H -norepinephrine, 4) rate of conversion of ^3H -tyrosine to ^3H -norepinephrine, 5) MAO activity, 6) COMT activity, 7) TH activity.

All vascular tissues will be carefully cleaned of adhering tissue with forceps or a small nylon brush as described by Koletsky et al (Proc. Soc. Exp.

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Biol. Med. 102: 12-15, 1959). Microscopic examination of the vessels will be made to confirm that adhering tissues (connective tissue, fat and extra-vascular nerves) have been removed.

METHODS

Animals will be killed by a blow on the head and decapitated. Various tissues will be rapidly removed, cleaned, frozen on dry ice and stored at 20°C prior to analysis.

Chemical Methods

1. Endogenous norepinephrine will be assayed by the method of Anton and Sayre (J.P.E.T. 133:360, 1962). The method involves the selective absorption of catecholamines onto a constant amount of aluminum oxide, elution with a constant volume of perchloric acid and their measurement by the formation of fluorescent trihydroxyindole in the presence of potassium ferricyanide and alkaline ascorbare. To differentiate between epinephrine and norepinephrine, fluorescence is measured at 2 different pHs (pH 2-3 and pH 5-7). In the lower pH range, norepinephrine compared to epinephrine has a negligible fluorescence. Of the naturally occurring analogues of norepinephrine, only dopamine interferes but this interference is reported to be relatively small. Samples will be run in duplicate and recovery rates of standard amounts of epinephrine and norepinephrine are calculated for each analytical run. Recoveries up to at least 75% from biological materials have been reported.
2. ³H-norepinephrine will be estimated by adding an aliquot of eluate (obtained after the alumina absorption of labelled amine as described above) in the counting solution (Instagel: Packard Instrument Co.) and the radioactivity will be determined in a Nuclear Chicago Scintillation counter.
3. ³H-catechol deaminated metabolites will be assayed by the method of Kopin et al (J. Biol. Chem. 236: 2109, 1961).
4. ³H-normetanephrine will be assayed by the method of Iversen et al (J.P. E.T. 150:173, 1965).
5. ³H-methylated deaminated metabolites will be estimated by the difference between the total radioactivity of the tissue extracts and the sum of other metabolites.
6. Serotonin and dopamine will be simultaneously measured along with norepinephrine according to the method of Fleming et al (Analytical Chem. 37:629, 1965).

Enzyme Studies

Tissue will be removed, cleaned, weighed and homogenized in 2.0 ml of ice cold .25M sucrose. An aliquot (10 ul) of the homogenate will be used for assay of monoamine oxidase activity. The remaining homogenate will be centri-

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fuged at 26000 g for 20 min. Aliquots of the clear supernatant fluid will be assayed for tyrosine hydroxylase, PNMT and COMT activities.

Monoamine oxidase activity will be assayed by measuring the conversion of ^{14}C -tryptamine to ^{14}C -indoleacetic acid as described by Wurtman and Axelrod (Biochem. Pharmacol. 12:1439, 1964).

Catechol-o-methyl transferase (COMT) will be assayed by measuring the formation of ^{14}C -metanephrine on incubation with (-) epinephrine and ^{14}C -methyl-s-adenosylmethionine as described by Axelrod (in Methods of Enzymology, Vol. 5, p. 748, 1959, New York Acad. Press).

Tyrosine hydroxylase activity will be assayed by the method of Levitt et al (J.P.E.T. 148:1, 1965) with modifications described by Mueller et al (J.P.E.T. 101:379, 1969).

Phenylethanol-N-methyl transferase activity will be assayed by the method of Axelrod (J. Biol. Chem. 237:1657, 1962) using normetanephrine as the substrate and ^{14}C -S-adenosylmethionine will serve as a methyl donor.

Synthesis of Acetylcholine

Synthesis of acetylcholine will be measured from the rate of conversion of ^3H -choline to ^3H -choline to ^3H -acetylcholine according to the method by Marchbank (Biochem Pharmacol. 18:1763-1766, 1969).

Synthesis of 5-hydroxytryptamine

The measurement of 5-hydroxytryptamine turnover rate in the rat brain from the conversion of ^{14}C -tryptophan to ^{14}C -5-hydroxytryptamine will be made according to the method by Lin et al (J. Pharmacol. exp. Ther. 179:232-238, 1969).

Synthesis of Norepinephrine in Isolated Tissues

The measurement of norepinephrine turnover rate will be made by the amount of ^3H -norepinephrine formed from the ^3H -tyrosine according to the method of Weiner and Rabadjija (J. Pharmacol. Exp. Ther. 160:61-71, 1968). Many of these methods are already operative in our laboratory. The others will be set up for the purposes of this investigation.

SIGNIFICANCE OF RESEARCH

The importance of careful base-line observations is an appropriate model for understanding the mechanism of action of nicotine and smoking on the central nervous system and cardiovascular system cannot be overemphasized. Only in this way can we distinguish between early and late changes, between primary and secondary effects and between fundamental and the incidental. The investigation proposes to study the effect of nicotine and smoking on the pattern of catecholamines, 5-hydroxytryptamine and acetylcholine and to correlate these changes

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with acquired behavior. Furthermore, we propose to determine the changes in the pattern of neurohormones and in the acquired behavior associated with withdrawal of nicotine or cessation of smoking.

It is the ambitious long-term aim of this project to work toward the achievement of such a breakthrough, or at least make a significant advance in understanding the effect of smoking on the central nervous system and in the cardiovascular system. We are more than hopeful that our efforts will aid in the elucidation of the mechanism of the action of nicotine on the central nervous system and thereby answer the question "Why do we smoke?"

Cigarette smoking has been implicated by epidemiological studies as one of the "major hazards to health in the United States." Not only has it been associated with respiratory diseases and disorders, but it has also been implicated in the development of cardiovascular diseases, particularly in cases of death due to coronary diseases. So far, no causal mechanism has been found to explain these statistical relationships. It is our belief that these studies promise to throw light on the mechanism of action of nicotine and smoking and may thus assist in the development of prophylactic measures and help place therapy on a more logical basis in the treatment of cardiovascular diseases. We are thinking in particular of patients with coronary heart disease.

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#884 CASTRO

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October 26, 1972

Grant application No. 884

Refected

To: The committee comprising Drs. Cattell, Gardner and Jacobson

Subject: Albert Castro, Ph.D., Papanicolaou Cancer Research Institute,
Miami
New application No. 884
"Nicotine in Blood: Detection by Radioimmunoassay"

History

This proposal was Case No. 131, and full application was encouraged.

The request is for \$48,545, plus two additional years.

Documents Submitted (attached)

1. Application (undated) received by CTR October 13, 1972
2. Letters from CTR grantees Una Smith and James W. Ryan (both dated October 2, 1972) pledging active collaboration with Castro.

Comment

Telephone conversations with Drs. Castro and Ryan indicate that Castro's move to Miami should be completed February 1973. Both were told that a February 1 starting date is unlikely.

Castro raised no objection to a July 1, 1973 starting date if he is funded. Ryan, with candid concern for cash flow into the Papanicolaou Institute, urgently requests an April 1, 1973 starting date if funded.

Castro's attention has been called to the NCI published request for contract proposals on "Nicotine Levels in Blood: Detection by Radioimmunoassay". He states he has obtained information on conditions of such contracts and finds them unacceptable.

F.W.N.
F.W.N.

FWN:jfr

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THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

COMMITTEE:

Dr. Cattell
Dr. Gardner
Dr. Jacobson

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

Application For Research Grants

OCT 13 1972

Date:

1. Name of Investigator(s): (include Title and Degrees)

Albert Castro, Ph.D., Senior Scientist

2. Institution &

Address:

Papanicolaou Cancer Research Institute
1155 N.W. 14th Street
Miami, Florida 33136

3. Short Title of Project: NICOTINE IN BLOOD: DETECTION BY RADIOIMMUNOASSAY

4. Proposed Starting Date: February 1, 1973

5. Anticipated Duration of this Specific Study: 3 years

6. Brief Description of Objectives or Specific Aims:

The major objective of this proposal is to develop means of quantifying nicotine and its major metabolites in blood at the levels achieved as a consequence of tobacco smoking. In view of the low levels of substances to be measured and in view of our experience in detecting hormones and drugs, we believe that radioimmunoassay is the procedure of choice. The research proposal covers the development of specific antisera against nicotine, cotinine, hydroxycotinine and desmethylnicotine. As antisera become available, protocols will be developed for assaying nicotine and its metabolites using "wet" chemistry techniques. However, we think that standard radioimmunoassay methodology can be simplified significantly by converting to "solid-phase." Specifically, as "wet" chemistry protocols are developed and field-tested, we will couple specific antisera to solid supports (e.g. silanized controlled pore glass), and solid-phase protocols will be developed and tested in parallel. Our preliminary experience with solid-phase radioimmunoassays of other substances (e.g. thyrotropin and progesterone) indicate that the time required for a given assay may be reduced seventy-fold.

7. Give a Brief Statement of your Working Hypothesis: Appended

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8. Details of Experimental Design and Procedures: (Attach Separate Pages) Appended

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

Appended

10. Additional Requirements:

Nuclear of Chicago 300-place, B-channel liquid scintillation counter

We plan to use ^3H -nicotine as the displacement tracer. Therefore liquid scintillation counting is essential to the proposed research project.

Animals: The projected field tests require large numbers of animals. Possibly these costs could be reduced if we could gain access to blood samples used in other field tests supported by CTR.

11. Biographical sketches of all principal and professional personnel (append) Appended

12. List of publications: (Five most recent as pertinent) (append) Appended

13. Budget: (1st year)

3.

A. Salaries (Personnel by names)	% time	Amount
Professional		
Albert Castro, Ph.D., Principal Investigator	30%	9,000
Alfred Chung, M.S., Organic Chemist	50%	7,000
Fringe Benefits (10% of salary) Includes FICA medical insurance, life insurance and unemployment		1,600
Technical		
Laboratory Technician	100%	6,000
Fringe Benefits (10% of salary)		600
Sub-Total		24,200
B. Consumable Supplies (list by categories)		
Animals: 100 rabbits @\$7		700
maintenance \$0.30/day/rabbit, average 30 days		900
Chemicals and glassware		600
Radioactive isotopes		1,400
Sub-Total		3,600
C. Other Expenses (itemize)		
Travel to Scientific Meetings, 1 trip		500
Sub-Total		500
D. Permanent Equipment (itemize)		
Nuclear of Chicago liquid scintillation counter		16,000
300-place, 3-channel		
		16,000
E. Overhead (15% of A+B+C)		4,245
Total		48,545

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	26,015	3,780	500	-0-	4,544	34,839
Year 3	27,966	3,969	500		4,865	37,300

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature *[Signature]*
 Director of Project 503/666-7959
 Telephone
 Signature *[Signature]*
 Business Officer of the Institution
 305/371-5572 Telephone

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Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current

Title of Project	Source	Amount	Duration
Non-renal renin-angiotensin systems in man	Oregon Heart Association	\$9,100	July 1971- July 1973
Studies of Renin-angio-aldosterone inter- relationship in acute saline depletion	Oregon Heart Association	10,376	July 1971- July 1972
Renin angiotensin system	Commack Foundation	7,800	July 1971- July 1972
Insulin release	Oregon Diabetes Association	2,000	July 1971- July 1972

ending

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7. Working Hypothesis

Depending on the dose and the route of administration, nicotine may stimulate or depress sympathetic and parasympathetic ganglion cells, the adrenal medulla, motor end plates of skeletal muscle, ciliated epithelial cells of the airways and chemoreceptors of the coronary, pulmonary, aortic and carotid arteries (for review see Comroe, 1960). This listing of pharmacologic effects is by no means exhaustive. Still the point remains that it is not known which if any of these pharmacologic effects play a role in the disease processes statistically linked with the use of tobacco and possibly with nicotine.

The basic problem is that little is known of the concentrations of nicotine and its metabolites achieved in blood, other body fluids and tissues as a consequence of smoking. We believe that modern assay technology can remedy this situation, and we therefore propose to develop radioimmunoassays for nicotine, cotinine, hydroxycotinine and desmethylnicotine. Emphasis will be placed on simplicity and rapidity of use as well as on specificity and sensitivity. We then plan to make these assays available to all interested investigators to permit a direct reexamination of the actions of nicotine and its metabolites as a function of inhalation of tobacco smoke.

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Background

As pointed out by Isaac and Rand (1969), nicotine is one of the most commonly used drugs. Furthermore, few drugs are administered in as complex a manner as is nicotine in tobacco smoke: generally eight or more small doses are taken by inhalation at roughly one minute intervals in the smoking of one cigarette, and the "addicted" smoker repeats this process at 15-30 minute intervals throughout the waking day.

The fate of the dose of nicotine in tobacco smoke is no less complicated as it enters the respiratory tract. Vaughan and Vaughan (1969) have drawn attention to the necessity for distinguishing between "deposition" and "retention" when studying the fate of inhaled aerosol particles. Nicotine arrives in the respiratory tract as part of an aerosol of semi-liquid, multi-component particles. Presumably some components of the aerosol may transfer rapidly from the particles to the blood stream depending on the chemical nature of the component itself. However, it is reasonable to believe that readily-absorbed substances such as nicotine may be taken up at variable rates depending on where the original aerosol lands.

It has been recognized for many years that studies of the absorption, actions and fate of nicotine must depend in large measure on highly-specific, sensitive means of detection of nicotine itself and its known and possible metabolites (McKennis et al., 1958, 1959, 1960 a & b). So far, the requirement for specificity has been met but the requirements for adequate sensitivity and range (in terms of metabolites) have not. McNiven et al. (1965) and Beckett and Triggs (1966) have reported selective extraction methods and protocols for detection by gas-liquid chromatography. However, 50 ng of nicotine begins to fall below the limits of detection. More recently, Isaac and Rand (1972) used an alkali flame ionization detector with gas-liquid chromatography and extended sensitivity to about 3 ng. Using the latter method, one can readily detect nicotine in blood immediately after smoking but levels become undetectable in a large proportion of subjects within two hours. Possibly of equal importance the method does not detect nicotine in the blood of heavy smokers after overnight abstinence. Of great practical importance, the extraction and detection methods are tedious and do not allow for the processing of large numbers of samples.

In contrast to the state of the art of measurement of nicotine, pharmacologic studies of the actions of nicotine have been carried out with rigor since the turn of the century. Even so, the situation described by Comroe in 1960 still exists: many physicians are familiar only with the effects of relatively large doses of nicotine applied locally or given intravenously. The questions remain, what does nicotine do in the doses inhaled in tobacco smoke, what are its usual metabolites and what concentrations are achieved in body fluids other than urine?

The recent successes in the development of radioimmunoassay of drugs, steroids and polypeptide hormones of body fluids in our laboratories (we now have assays for phenobarbital and morphine) indicates the feasibility of developing similar assays for nicotine and its major metabolites, cotinine, hydroxycotinine and desmethylnicotine (McKennis et al., 1958, 1959, 1960 a & b). In principal, we should be able to achieve sensitivities within the picogram range.

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We propose to develop these assays as a first step toward answering the question, what concentrations of nicotine and its metabolites are achieved in body fluids when nicotine is administered as a component of inhaled tobacco smoke? As indicated by results of preliminary studies, the assays can be converted to solid-phase protocols and can be automated as described in this proposed project. As suitable assay protocols are developed, we plan to make the protocols and the relevant antibodies available to all interested investigators.

Specific Aims

1. Develop specific conjugates of nicotine and its metabolites suitable for producing antibodies.
2. Evaluate the antibodies in terms of their specificities.
3. Work out "wet" chemistry protocols for the relevant radioimmunoassays.
4. Examine the suitability of the protocols for use in solid-phase.
5. Field test the final assay protocols.

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METHODS OF PROCEDURE

1. Conjugation of Nicotine and Related Substances to Protein

The chemical structures of nicotine and its metabolites are shown in Fig. 1. Since cotinine, hydroxycotinine and desmethylnicotine are structurally similar, it is highly desirable to produce antisera having no serious cross-reactions.

In the case of nicotine, we propose to couple via position-6 of the pyridine ring. Alternative procedures are shown in Fig. 2: firstly, 6-aminonicotine will be obtained by reacting sodium azide with nicotine. The product will be reacted with succinic anhydride to form its hemisuccinamide. The latter product can be coupled to bovine serum albumin (BSA) or to bovine gamma globulin (BGG) using carbodiimide.

Alternatively, 6-aminonicotine will be hydrolyzed to 6-hydroxynicotine (Chichibabin and Burkholz, 1968), which in turn can be converted to 6-(4-aminobutoxy)-nicotine by reaction with N-(4-bromobutyl)-phthalimide and subsequent acid hydrolysis. Again, coupling can be effected with carbodiimide.

We anticipate that by coupling via the pyridine ring, antibodies to nicotine and its major metabolites can be obtained with highly specific reactivities. Should this not be the case, coupling reactions applicable to the pyrrolidine ring will be used. In the latter reactions, we will start with nornicotine reacted with 4-bromobutyronitrile, 4-bromocrotonate or succinic anhydride followed by hydrolysis. The products are amenable to condensation to a carrier such as thyroglobulin using carbodiimide.

2. Production and Evaluation of Antibodies

As a routine, each conjugate will be injected into fifteen rabbits. We plan to use 1 mg of hapten-protein conjugate emulsified in 1 ml of 50% complete Freund's adjuvant in saline per injection per rabbit. Weekly injections will be made through the first month and then biweekly injections during the second month. The rabbits thereafter will receive monthly injections. In our experience, three to six months are required for good antisera.

Titration will be performed using serial dilutions. Affinity constants will be determined in the usual manner. Cross-reactivities will be determined using nicotine and its above-listed metabolites.

3. Assay Protocols and Sample Preparation

The proposed procedure for preparing samples is shown in Fig. 3. Should all of the antisera have serious cross-reactivity problems, a further separation will be effected using thin-layer chromatography.

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The nicotine levels in the blood of smokers has been reported to be 0.5-60 ng/ml of plasma (Isaac and Rand, 1972), depending on the smoking habits of each individual and the time elapsed after smoking. Since the efficiency of ether extraction of nicotine is greater than 90% (Isaac and Rand, 1969, 1972; Beckett and Triggs, 1966), the nicotine of the ether extract should be well above the anticipated minimum detectable level (20-30 pg/ml).

The addition of NaOH to plasma assures that nicotine and most of its metabolites will occur as free bases (nicotine: $pK_{a1} = 3$, $pK_{a2} = 8$ according to McKennis, 1960a). The possible exception is cotinine.

Twenty plasma samples, each from ten different plasma pools, will be prepared to contain serial concentrations of nicotine to be tested along with standard nicotine solutions to determine a) percent recoveries and b) the precision of the assays.

A Latin square design will be used as a preliminary evaluation of the radioimmunoassays.

e.g.

Test	Time			
	I	II	III	IV
1	B	C	A	D
2	C	A	D	B
3	D	B	C	A
4	A	D	B	C

Fifty plasma samples with four dose levels A, B, C and D will be given to four different researchers 1, 2, 3 and 4 at different times I, II, III and IV for the assay of nicotine and then its metabolites. The researchers will not know the dose levels with which they are working but will be given a series of nicotine standard solutions.

Theoretical considerations. The principle of radioimmunoassay is based on the competition of labelled and unlabelled antigens for specific antibody binding sites (Yalow and Berson, 1967; Odell and Daughaday, 1971).

In principle, the sensitivity of a radioimmunoassay can be evaluated from the following equation (See Appendix I-A):

$$\Delta C_H = (-\Delta b/b) \left[(C_H^* + C_H) (1-b)^2 + 1/k \right] / (1-b)^2 \quad (0 \leq b \leq 1) \quad (1)$$

In which K , C_H^* , C_H , and b are respectively the affinity constant of the antiserum, the concentrations of labelled and unlabelled haptens, and the fraction of the labelled hapten bound to the antibody.

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At minimum unlabelled hapten concentration, Eq. (1) becomes

$$\Delta C_H \doteq (-b/b) \left[C_H^* (1-b)^2 + 1/k \right] / (1-b)^2 \quad (0 \leq b \leq 1) \quad (2)$$

This means that the minimum detectable hapten C_H is determined by the affinity constant of the antiserum, K , the concentration of the labelled hapten C_H^* used, and the change in bound hapten, Δb , due to the addition of C_H in the dilution of antiserum is such to give a fraction of bound hapten b .

Furthermore, if the affinity constant of the antiserum is sufficiently high to meet the criterion

$$1/K \ll C_H^* (1-b)^2 \quad (0 \leq b \leq 1) \quad (3)$$

Eq. (2) would become

$$C_H = C_H^* (-\Delta b/b) \quad (0 \leq b \leq 1) \quad (4)$$

The labelled nicotine available from commercial source at the present time has a specificity of 100-250 mc/m mole, equivalent to $22.2-55.5 \times 10^{10}$ DPM/m mole. Therefore, at least 650 pg of labelled nicotine (C_H^*) would be needed to get at least 1000 cpm in tests, if a 50% counting efficiency is assumed for counter.

The change in percentage bound, $(\Delta b/b \times 100)$ could be considered to be significant only if the Δb is at least twice the standard deviation of b . That is, $(\Delta b/b \times 100)$ has to be at least twice the coefficient of variation of the measurement of b . The minimum detectable nicotine estimated from Eq. (4) would be $C_H = 650 \times 0.04 = 26$ pg, if the coefficient of variation is 2%.

Thus, the sensitivity of the radioimmunoassay of nicotine will be estimated at about 30 pg (95% confidence), if the specific activity of labelled nicotine is higher than 250 mc/mmole and the affinity constants of the antisera meet the criteria (3). The latter requires the affinity constant $K \gg 10^8 \text{ M}^{-1}$.

The affinity constant of an antiserum at about 10^{10} M^{-1} seems to be common in antisera for steroids (Odell and Daughaday, 1971). The antisera against barbiturates and morphine also show affinity constants at the same order of magnitude. This indicates that the affinity constants of nicotine antisera may be also in this range.

A higher sensitivity could be achieved if labelled nicotine with a higher specific activity were available. On the other hand if the affinity constant of the antisera for nicotine could not meet the criteria (3), the sensitivity of this radioimmunoassay would be expected to be lower.

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4. Solid-Phase Protocols

Recently means have been found for immobilizing biological materials to insoluble carriers. In the case of antibodies, immobilization by covalent bonding can be carried out without affecting specific binding capacities (Messing, 1970; Weetall, 1969, 1970 a & b). On the basis of our preliminary experience with solid phase radioimmunoassays of thyroid stimulating hormone and progesterone, it appears that solid-phase antibodies can be used to reduce apparent antigen-antibody equilibrium times and therefore elapsed assay times by as much as seventy-fold.

Methods of insolubilization. Our experience so far has been with inorganic supports, in particular with silanized controlled pore glass (Corning Glass Works). We prefer inorganic supports because they are reusable, resistant to microbial contamination, are of high specific activity (and therefore are readily removed from suspension by centrifugation) and have sufficient intrinsic strength for use in columns developed under pressure. The major organic alternative, Agarose, is inferior in comparison with each of these points. Furthermore, in view of the large number of substituted silane reagents available, it is our opinion that a wider variety of derivatizing reactions can be used with silanized inorganic supports than with dextran gels or other organic polymers. There is the further point that inorganic carriers, once sized, do not fracture readily and therefore do not require repeated size characterizations.

With existing inorganic supports and silane reagents, it is possible to bind antibodies using acid chlorides, carbodiimide, diazo-reagents and isothiocyanate.

Antisera to nicotine and its metabolites will be coupled to controlled pore glass (mesh size 120/200, mean pore diameter 500 A, pore vol. 0.84 ml/g) silanized with γ -aminopropyl-triethoxysilane to produce an alkylamine glass. The alkylamine glass can be used directly or can be converted to an arylamine glass by refluxing with triethylamine and p-nitrobenzoylchloride. Glass beads of the dimensions described above are heavy enough to be removed by brief centrifugation yet are fine enough to resist rapid sedimentation by unit gravity. These properties give excellent control over the exposure of binding-protein to antigen and discrete termination of exposure. Assay protocols will be based on the specific wet chemistry protocols described previously.

5. Field Tests

In order to establish reproducible routine procedures for the radioimmunoassays of nicotine and its major metabolites, animal experiments will be performed as follows:

a. Sixty male and sixty female rabbits will be divided into 12 groups as shown in the following table:

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Dose (ug)	Male		Female	
	Young	Adult	Young	Adult
1	10	10	10	10
2	10	10	10	10
4	10	10	10	10

Rabbits 3-4 weeks and 4-5 weeks of age will be grouped as "young" and "adult." In these experiments, nicotine will be administered by a single intravenous injection. These dose levels correspond to the blood nicotine levels after smoking 1/2 to 2 cigarettes. Seven 4 ml samples of blood will be taken from each rabbit at 2, 4, 8, 16, 32, 64 and 128 minutes after the injection and will be tested for nicotine by radioimmunoassay and by gas-liquid chromatography.

b. In the second test, 160 male and 160 female rabbits will be divided into 16 groups as shown below:

Dose (ug)	Male		Female	
	Young	Adult	Young	Adult
2	20	20	20	20
4	20	20	20	20
6	20	20	20	20
8	20	20	20	20

These doses correspond to the nicotine levels achieved in blood after 1 to 4 cigarettes. Nicotine will again be administered by a single intravenous injection once every two weeks. Blood samples will be taken twice, once at one hour and the other at one week after injection. In order to handle these large numbers of animals, each group will be divided into five subgroups, such that 20 rabbits can be handled 4 at a time in five week days. The blood samples will be tested by radioimmunoassay and gas-liquid chromatography.

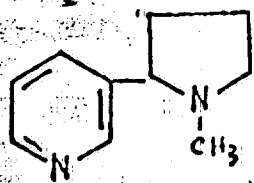
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SIGNIFICANCE

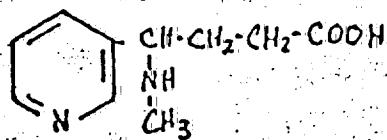
This project is aimed at the development of radioimmunoassays of nicotine, cotinine, hydroxycotinine and desmethylnicotine to answer the question, what levels of these compounds are achieved in body fluids as a consequence of smoking? Through the development of these assays, we then plan to make the protocols and antisera available to all interested investigators to allow reexamination of the question, what are the actions of nicotine and its metabolites in man in the doses inhaledⁱⁿ tobacco smoke?

Existing assay methodologies are too insensitive to detect nicotine in the concentrations likely to occur in blood one or two hours after smoking. In addition, virtually nothing is known of the immediate fate of nicotine or of the concentrations of its metabolites achieved in body fluids other than urine. The metabolites, if they occur in blood, may well be of interest not only in terms of carcinogenesis (e.g. aromatic amines and cancer of the bladder) but also in terms of nicotinic effects on cardiac conduction, sympathetic and parasympathetic synaptic events, tracheal epithelial ciliary motion and neuromuscular transmission. However, we believe that investigations of these possible actions should await a clear understanding of the concentration of nicotine and its metabolites likely to arrive at possible target sites. Clearly, the magnitude of possible physiologic and pharmacologic experiments exceeds our expertise and available manpower. Therefore the emphasis of this project is on developing and making available the materials and protocols necessary for highly sensitive, specific and simple assays.

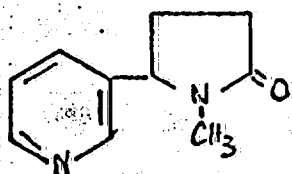
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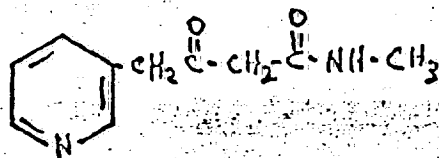
(-)-nicotine



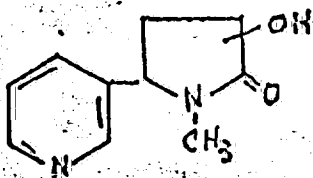
(+)- α -3-(pyridyl)- α -methylaminobutyric acid



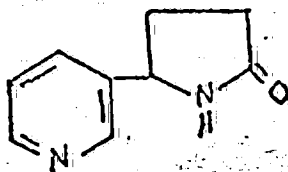
(-)-cotinine



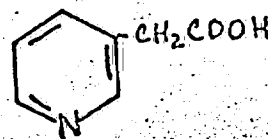
α -(3-pyridyl)- β -oxo-N-methylbutyramide



Hydroxycotinine



(-)-desmethylnicotine

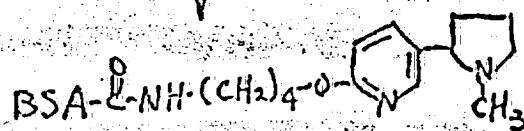
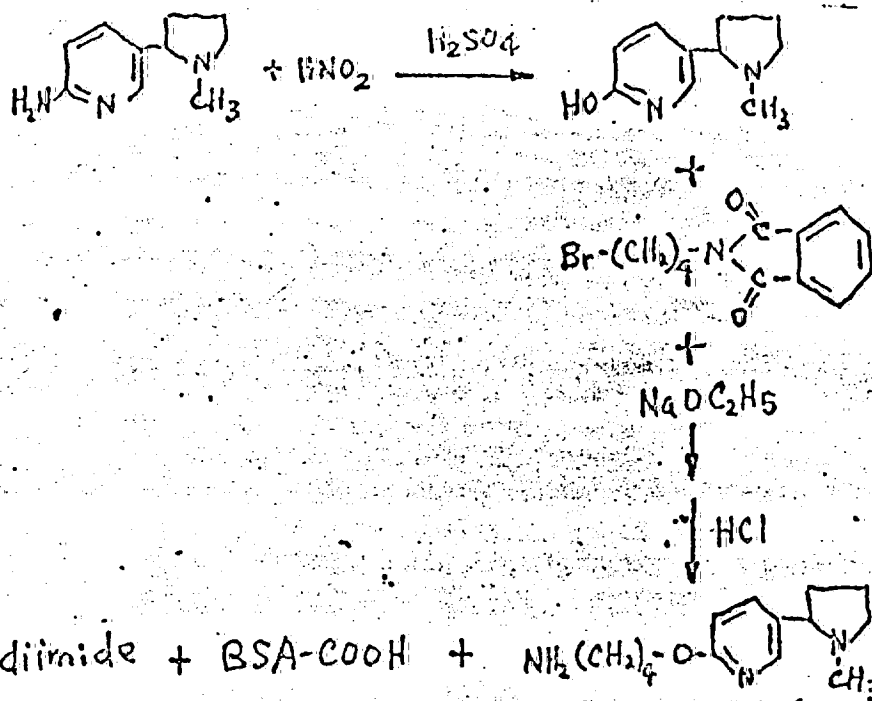


3-pyridylacetic acid

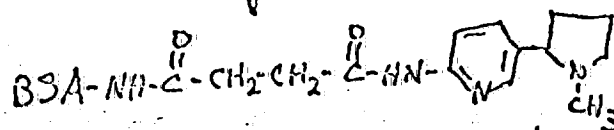
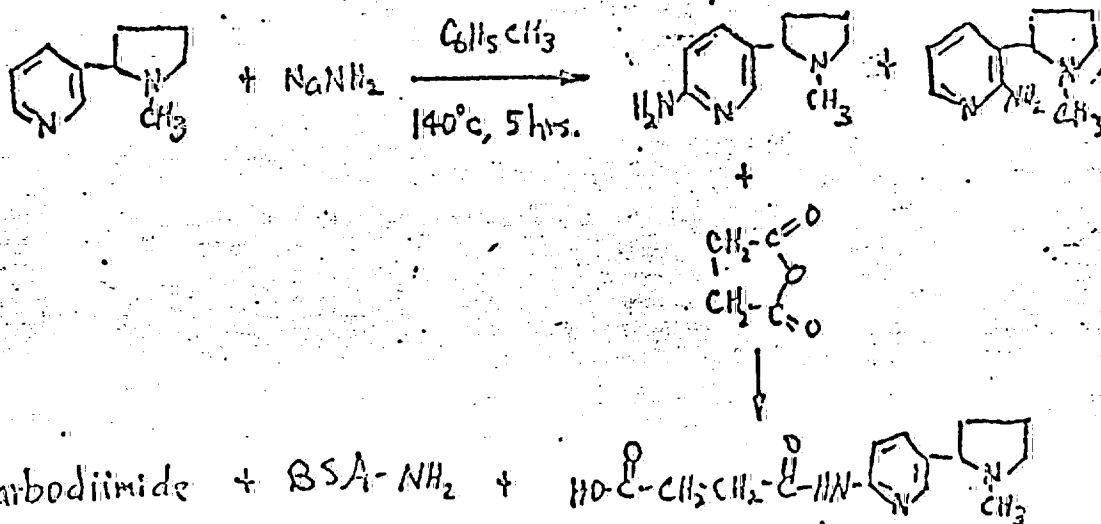
Figure 1. Major Nicotine Metabolites

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1.



2.



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Figure 2. Synthesis and Conjugates of Nicotine to BSA

0.1 ml plasma (contains 0.05 to 6 ng nicotine)

+ 0.02 ml 5M NaOH

3 x 0.5 ml ether extraction

1.2 ml of ether extract (in a 8 x 75 mm tube)

evaporate to dryness

+ 0.1 ml of labeled nicotine - H^3 (x 1500 cpm) buffer solution

+ 0.1 ml of nicotine antiserum buffer solution

Incubate overnight at 4°C

+ 0.2 ml of saturated $(NH_4)_2SO_4$ solution

Centrifuge at 3500 rpm for 20 min. at 4°C

0.2 ml supernant in a counting vial

+ 10 ml scintillation fluid

Shake for 10 minutes

Count free nicotine - H^3

Compare with standard curve

Calculate nicotine content

This could be circumvented if the specificity of nicotine antiserum is high.

Figure 3. Proposed procedure for determination of nicotine in blood by radioimmunoassay.

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The GLC method reported by Isaac and Rand (1972) will be used in this study.

2.5 ml plasma

+3 ml of 15 ug/ml modaline soln

+1 ml of 5 M NaOH

3 x 5 ml ether extraction

about 11 ml ether extract

evaporated on 42°C water bath

10 ul ether extract

2 ul ether extract injected to GLC

Calculate the nicotine content

Figure 4. Detection of Nicotine by GLC

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Facilities

1. Space. The space will be comprised of 5000 sq. ft. with facilities for high and low levels of radioactivity, counting room, labelling room, electron microscope room, peptide synthesis and amino acid analyzer, general lab, pump room, organic lab, cold room, data room, conference room, and offices. This space will be shared with Doctors J.W. Ryan and Una Smith, Senior Scientists of the Papanicolaou Cancer Research Institute. Rabbits are boarded in the Central University facility.

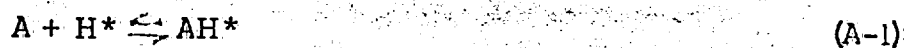
2. Major Items of Equipment

Philips EM 100 electron microscope
LKB knifemaker
Durst enlarger and ancillary preparative and photographic equipment
Zeiss binocular microscope with camera and phase contrast attachments
Gilson and LKB fraction collectors
Craig-Post 200-transfer countercurrent distributor
Spinco 120C amino acid analyzer
Unilux II liquid scintillation counter
Actigraph strip counter
Gilford-Beckman recording spectrophotometer
Buchi flash evaporator
Grass four-channel polygraph
Radiometer automatic titrator
Radiometer pH meters (2)
Mettler top pan balance
Sartorius analytical balance
Apparatus for cleavage with anhydrous HF
Apparatus for paper and thin layer chromatography and electrophoresis
Pumps for infusion and respiration of small animals
Vacuum oven
Polarimeter
Circulating water baths and incubators
Apparatus for manual and automatic synthesis of polypeptides
Virtis lyophilizer
Two channel auto analyzers
Sowall RCB-2 centrifuge
Two respirators (one cat, one rabbit-cat)

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Appendix A - Derivation of Equation (1)

From the references (15-17), the equilibrium of hapten, antibody, and antibody-hapten system can be expressed as:



where A, H, H*, AH, and AH* are respectively antibody, unlabeled and labeled haptens, and unlabeled- and labeled-antibody complexes. The affinity constants (apparent equilibrium constants) for antibody would be

$$K_H^* = [AH^*] / [A][H^*] \quad (A-3)$$

$$K_H = [AH] / [A][H] \quad (A-4)$$

The mean the concentrations.

The material balances are:

$$C_A = C_0/n = [A] + [AH^*] + [AH] \quad (A-5)$$

$$C_{H^*} = [H^*] + [AH^*] \quad (A-6)$$

$$C_H = [H] + [AH] \quad (A-7)$$

where C_A , C_{H^*} , AND C_H are respectively the total concentrations of antibody labeled, and unlabeled haptens, and C_0 is the initial antibody concentration

in antiserum prior to an n-fold dilution. Eqs. (A-3) and (A-4) may be rewritten as:

$$[A] = [AH^*] / K_H^* \cdot [H^*] \quad (A-8)$$

$$\text{and } [AH] / K_H \cdot [A] \quad (A-9)$$

From Eqs. (A-7) and (A-9), one can obtain:

$$AH = \frac{C_H}{1 + K_H [A]} \quad (A-10)$$

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Substitution Eq. (A-8) to Eq. (A-10) gives:

$$[AH] = \frac{C_H}{1 + K_H^* [H^*] / [AH^*]} \quad (A-11)$$

If the antibody does not discriminate against either labeled hapten or unlabeled hapten, K_H^* would be equal to K_H .

Let $K = K_H^* = K_H$, $b = [AH^*] / C_H$, and $f = [H^*] / C_H^*$, then from eqs. (A-8), (A-11), and (A-5) one obtains:

$$C_A = b/Kf + C_H^* + C_H) b \quad (0 \leq b \leq 1) \quad (A-12)$$

$$C_A = b/K (1-b) + (C_H^* + C_H) b \quad (0 \leq b \leq 1) \quad (A-13)$$

Differentiation of Eq. (A-13) results:

$$dC_H/db = \left[1/K + (C_H^* + C_H) (1-b)^2 \right] / b (1-b)^2 \quad (A-14)$$

This can be rewritten as:

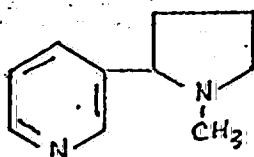
$$dC_H = (-b/b) \left[1/K + (C_H^* + C_H) (1-b)^2 \right] / (1-b)^2 \quad (0 \leq b \leq 1) \quad (A-15)$$

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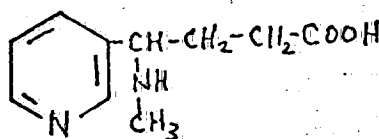
Appendix A-2

NICOTINE ALBUMIN CONJUGATE

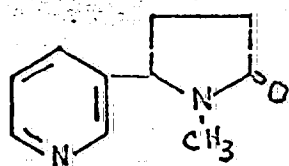
The major nicotine metabolites have been identified as (+) α -3-(pyridyl) α -methylaminobutyric acid. (-)-cotinine, β -oxo-N-methylbutyramide, hydroxycotinine, (-) desmethycotinine and 3-pyridylacetic acid with the following structure:



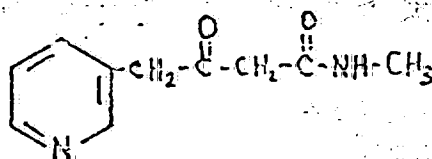
(-)-nicotine



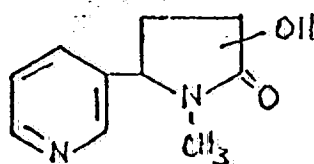
(+)- α -3-(pyridyl)- α -methylaminobutyric acid



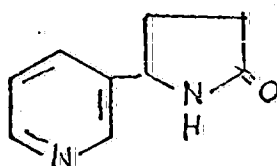
(-)-cotinine



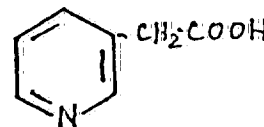
α -(3-pyridyl)- β -oxo-N-methylbutyramide



Hydroxycotinine



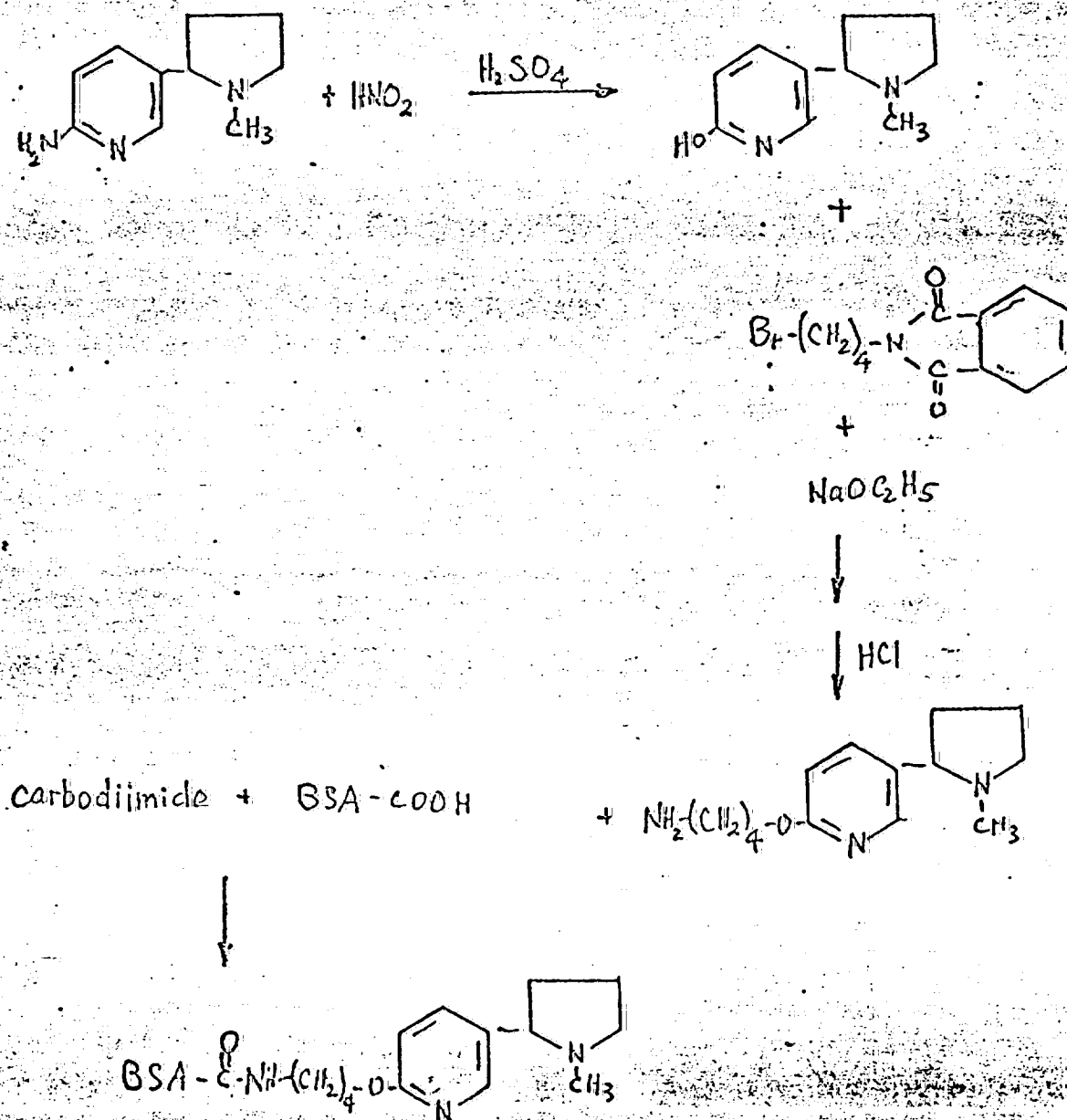
(-)-desmethycotinine



3-pyridylacetic acid

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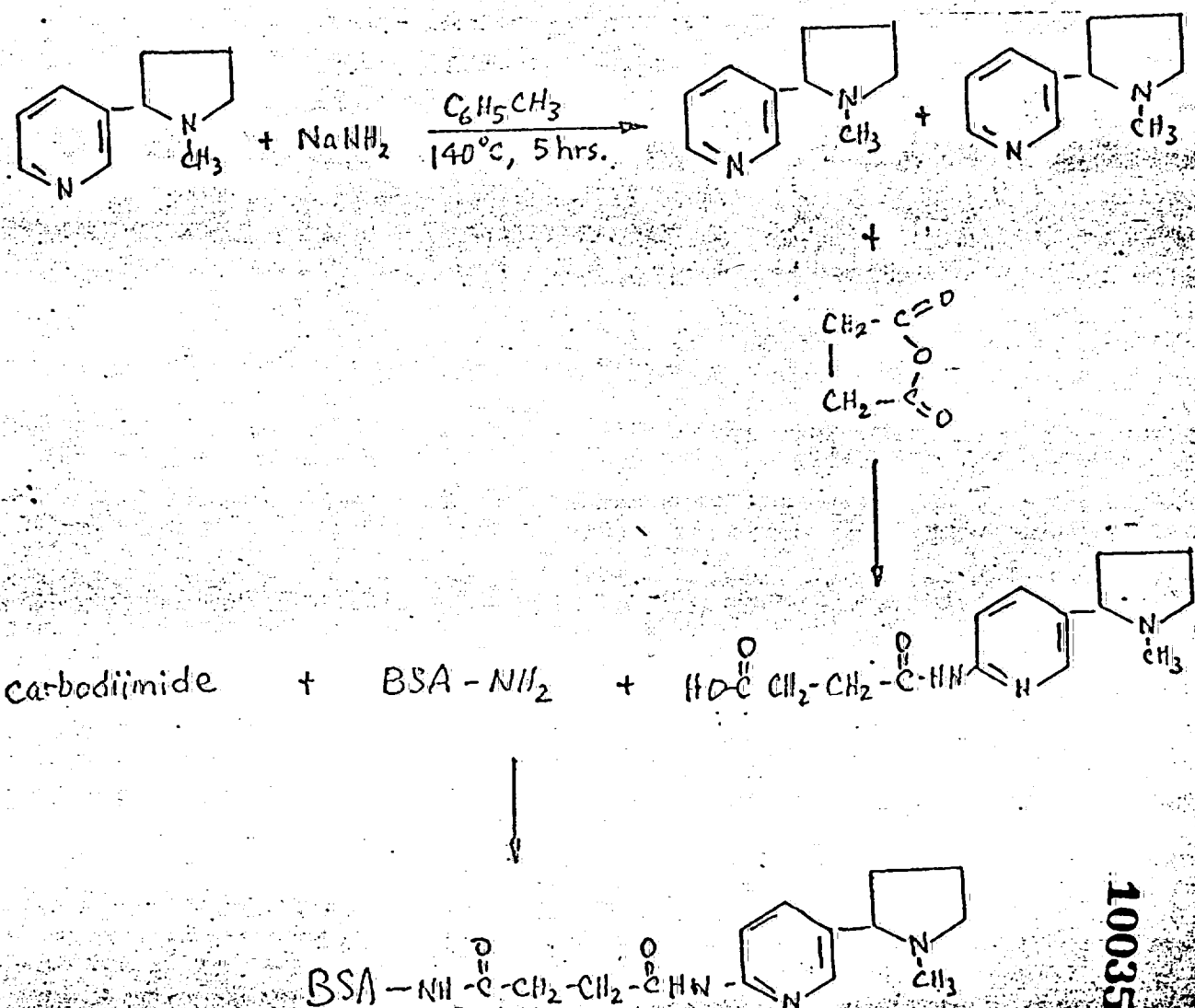
We have found that some of the compounds consisting of both amino and acid group in the same molecule can form a strong intramolecular hydrogen bond and hence interfere with the hapten albumin conjugation. Alternatively we propose to synthesize 6-hydroxynicotine by the action of HNO_2 in the presence of H_2SO_4 . The sodium salt of 6-hydroxynicotine can then be reacted with N-(4-bromobutyl)-phthalimide; and the free amine formed upon hydrolysis.



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It is well known that the antigenic sensitivity and specificity is predominantly directed toward the distal portion of the hapten. It seems reasonable to conjugate the nicotine to albumin through either five or six position of the pyridyl ring in order to distinguish nicotine from its metabolites.

Nicotine is first reacted with sodium amide to produce 6-amino nicotine. The product subsequently reacted with succinic anhydride and then conjugated to albumin.



1003538836

Biographical Sketch

Albert Castro

Principal Investigator

b. November 15, 1933, San Salvador, El Salvador
Central America

Permanent resident,
U.S.

Major research interest: Hormonal regulation of blood pressure and its
pathological disturbance in hypertension

Education:

<u>Institution and Location</u>	<u>Degree</u>	<u>Year Conferred</u>	<u>Scientific Field</u>
University of Houston, Houston, Texas	B.S.	1958	Biology
Baylor University, Jeff Davis Hosp., Houston, Texas	M.T.	1958	
University of El Salvador, El Salvador, C.A.	Ph.D.	1962	Biological Chemistry

Honors:

PHS Senior Postdoctoral Fellowship, 1965-1969.
Award and Member, ATENEO, Academy of Science of El Salvador
General Relator, 3rd International Congress of Dental Education
(127 universities) Mexico, 1964.
Northwest Pediatric Research

Professional experience:

- 1969---- Assistant Professor of Pediatrics and Co-Director, Pediatric Renal-Metabolic Laboratory, University of Oregon Medical School, Portland, Oregon.
- 1968-70 Senior Research Fellow, Diabetes and Metabolism. University of Oregon Medical School, Portland, Oregon.
- 1966-68 Postdoctoral Research Fellowship, Diabetes and Hypertension. University of Oregon Medical School, Portland, Oregon.
- 1967 Course in Radioisotopes in Medicine, University of Oregon Medical School, Portland, Oregon, under Dr. E. Hine, August, 1967.
- 1963 Course in Gas and Thin Layer Chromatography, Department of Chemistry, University of El Salvador. Under Professor J. Kannan.
- 1958-60 Assistant Professor of Microbiology and Biochemistry, Dental and Chemistry Schools, University of El Salvador, C.A.
- 1960-63 Associate Professor of Biochemistry and Microbiology, Dental and Medical Schools, University of El Salvador, C.A.
- 1965-66 Professor and Head, Department of Basic Sciences, Dental School, University of El Salvador, C.A.
- 1965-66 Co-Director of Graduate Research, Faculty of Chemistry, University of El Salvador, C.A.

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Publications Relevant to this Proposal:

1. Bailey, R.E., Brice, L.T., Castro, A., Lockwood, D.: Juvenile mineralocorticoidism with adrenal hyperplasia (AH)-an unusual cause of hypertension. Clin. Res. 16: 146, 1968.
2. Bailey, R.E., Brice, L.T., Castro, A., Lockwood, D.: Juvenile mineralocorticoidism with adrenal hyperplasia (AH) - an unusual cause of hypertension. Northwest Society Clin. Res., Vancouver, B.C., Jan. 1968. Proceedings of 3rd International Congress of Endocrinology, Mexico City, June, 1968.
3. Castro, A., Scott, J., Brice, L., Belts, R., Kimberling, W., Bailey, R.E.: The plasma glucose (PG) response to different oral glucose loads in healthy subjects. IIIrd International Congress of Endocrinology, Mexico City, June, 1968. American Diabetes Association, 28th annual meeting, San Francisco, June 1968. Diabetes 17: 319, 1968.
4. Bailey, R.E., Brice, L.T., Kramer, R., Macfarlane, D., Bartosova, D.: Periodicity in the "Ectopic ACTH Syndrome" a new finding. Clin. Res. 17:140, 1968.
5. Castro, A., Scott, J.P., Grettie, D., Macfarlane, D., Bailey, R.: Plasma insulin and glucose response of healthy subjects to varying glucose loads during three hour oral glucose tolerance test. Diabetes 19:842, 1970.
6. Bailey, R.E., Castro, A., Kramer, R.M., Macfarlane, D.: Enhancement of insulin release to acute glycemic stimulation with depression of basal insulin production rates in insulinoma following diazoxide administration. Acta Endocrinologica 63: 392, 1970.
7. Buist, N.R., Campbell, R., Castro, A. and Brent, B.: Congenital insulinoma producing hypoglycemia in the newborn. Pediatrics 47:605, 1971.
8. Castro, A., Kline, W.B., Cyderman, W.G.: A semi-automatic device for dispensing absorbent materials in protein bound assay. Steroids 15:641, 1970.
9. Castro, A. and Grettie, D.: Effect of diazoxide administration on plasma glucose and insulin secretion during two successive 100 gm glucose tolerance and IV tolbutamide. VIIth Congreso Panamericana de Endocrinologia, Sao Paulo, Brazil, 1970.
10. Price, L., Stone, G., and Castro, A.: Perspectives in thyroid testing. Lab News, p. 12-23, March, 1971.
11. Castro, A., Buist, N.R. and Dyess, K.: The insulin/glucose ratio during hypoglycemia: a new phenomenon on the diagnosis of hormone producing tumors. (In press) Diabetes, 1972.
12. Hammond, R., Dyess, K., and Castro, A.: Tn production and glucose tolerance in patients with granuloma annulare. (In Press) British J. of Dermatology, 1972.
13. Castro, A., Bartos, D., Bartos, F., Grettie, D. and Stone, G.: A practical guide in the diagnosis of hypertension. Lab News, February 1972.
14. Bailey, R.E., Bartos, D., Bartos, F., Castro, A., Dobson, R., Grettie, D., Kramer, R., Macfarlane, D. and Sato, K.: Rapid activation of aldosterone production and the renin angiotensin system by acute thermal stress. Experientia 28:159, 1972.
15. Castro, A., Bartos, F., Jowell, J., Grettie, D., Bartos, D., Stone, G. and Kondrasky, K.: A semiautomatic device for drying samples in steroid radioimmunoassay. Steroids 19:59, 1972.
16. Chung, A., Bartos, D., Bartos, F., Grettie, D. and Castro, A.: Radioimmunoassay of desoxycorticosterone (DOC). Clin. Res. 20:175, 1972.
17. Alvarez, L.C., Dimas, C.O., Castro, A., Rossman, L.G., Vanerlaan, E.F. and Vanerlaan, W.P.: Growth hormone in malnutrition. Clin. Res. 20:163, 1972.

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18. Castro, A.: Application of radioimmunoassay testing to disease predictability. *Ann. N. Amer. Med. & Dent. Assoc.* 5:10, 1972.
19. Alvarez, L.C., Dimas, C.O., Castro, A., Rossman, L.G., Vanderlaan, E.F. and Vanderlaan, W.P.: Growth hormone in malnutrition. *J. Clin. Endocr.* 34:400, 1972.
20. Kutas, M., Chung, A., Bartos, D. and Castro, A.: A progesterone radioimmunoassay without column chromatography. (In press) *Steroids*, 1972.
21. Castro, A., Bartos, D., Kutas, M. and Weiss, G.: A new two-piece micro-column in steroid preparation for radioimmunoassay. (In press) *Steroids*, 1972.
22. Stevens, J.W. and Castro, A.: Lipoatrophic diabetes (Submitted) *Diabetes*, 1972.
23. Castro, A.: Further experiences in islet cell adenomas. *J. Int. Acad. Meta.* 1:5, 1972.
24. Castro, A., Dyess, K., Buist, N., Kondrasky, K. and Potts, J.: The effect of diazoxide in children with islet cell adenomas. (Submitted) *Acta Endocrinologica Latino Americana*, 1972.
25. Castro, A., Bartos, F., Bartos, D., Grettie, D., Bailey, R.E.: A Practical approach to the production of angiotensin I and angiotensin II antibodies. (In press) *Biochem. Med.*, 1972.

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UNIVERSITY OF MIAMI

MIAMI, FLORIDA 33152

Mailing Address:
DEPARTMENT OF MEDICINE
SCHOOL OF MEDICINE
P. O. BOX 875, BISCAYNE ANNEX

Location:
MEDICAL RESEARCH BUILDING
1600 N. W. 10th AVENUE

October 2, 1972

Dr. Albert Castro
Co-Director, Metabolic Unit
Department of Pediatrics
University of Oregon School of Medicine
Portland, Oregon 97201

Dear Al:

I have read your project with great interest. I will of course be glad to supply any electron microscopy services which the project may require. We would be most interested to examine the lungs of rabbits used in your projected field tests. As the project develops we would particularly look forward to an investigation of the endothelial and epithelial components of the lungs of rabbits which have been exposed to cigarette smoke.

With best wishes for your studies

Yours sincerely,

Una Smith

Una Smith, Ph.D.,
Senior Scientist, Papanicolaou Institute,
Assistant Professor of Medicine,
University of Miami

US/srl

1003538840

UNIVERSITY OF MIAMI
MIAMI, FLORIDA 33152

Mailing Address:
DEPARTMENT OF MEDICINE
SCHOOL OF MEDICINE
P. O. BOX 875, BISCAYNE ANNEX

Location:
MEDICAL RESEARCH BUILDING
1600 N. W. 10th AVENUE

October 2, 1972

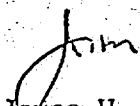
Dr. Albert Castro
Co-Director, Metabolic Unit
Department of Pediatrics
University of Oregon School of Medicine
Portland, Oregon 97201

Dear Al:

We very much look forward to your move to Miami. This letter is to confirm our previous verbal agreements. We can supply you with laboratory and office space. As you know, our unit now occupies the Sieron Building, which contains about 5,000 square feet of improved office and laboratory space. We can provide one office (about 100 sq. ft.) and two laboratory rooms (about 250 sq. ft each) for your high radioactivity and low radioactivity work. The rest of the laboratory space is for general use. Mr. Chung might expect to use our existing organic chemistry lab, and you and I will share the cold room, biochemistry labs and the vivarium.

Please let me know if I can be of any further assistance.

Best regards,


James W. Ryan, M.D., D.Phil.,
Senior Scientist, Papanicolaou Institute,
Associate Professor of Medicine,
University of Miami

JWR/srl

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#629D ERICSON

1003538842

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

January 31, 1973

Grant Application No. 629D

To: The committee comprising Drs. Bing, Cattell and Sommers

Subject: Carlton K. Erickson, Ph.D., University of Kansas, Lawrence
Continuation application No. 629D
"Effects of Nicotine on Free Acetylcholine in the Hippocampus
During Learning"

History

Since 1967, CTR has supported this and related lines of investigation by Dr. Erickson. The present application is in CTR parlance a "continuation", meaning a request for extension of support having no priority in competition.

Hence this application competes on the same basis as a new application. The heading on page 1 of the application was altered by the applicant.

The request is for \$11,891, for one year only. The last grant in the three-year series now ending was \$10,550 per year.

Documents Submitted (attached)

1. Application dated January 22, 1973.
2. Progress report, June 1, 1972 - December 31, 1972.
3. Manuscript "Alteration of Cortical and Reticular . . .", Erickson and Graham, in press, J. Pharmacol. exp. Therap. 1973.

Comment

Dr. Erickson promises to inform us in February of the outcome on the NIMH Special Fellowship for work in Sweden which is central to his plans for the ensuing year. In his application for this award he makes extensive reference to his nicotine work and CTR support. A copy (barely legible) is available here.

JWN
F.W.N.

FWN:wg
Encls.

10035388A3

Comm.
Dr. Bing
Dr. Cattell
Dr. Sommers

PHARMACOLOGY

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

Application for Renewal of Research Grant
~~Application for Research Grant~~

#629D
#629C-6/1/72-5/31/73
#629BR1-6/1/71-
5/31/72
#629B-6/1/70-5/31/71
#629A-11/1/68-10/31/
69
#629-11/1/67-10/31/68

JAN 29 1973

Date: January 22, 1973

1. Name of Investigator(s): (include Title and Degrees)

Carlton K. Erickson, Associate Professor of Pharmacology and Toxicology, B.S., M.S., Ph.D.

2. Institution &

Address: School of Pharmacy
University of Kansas
Lawrence, Kansas 66044

3. Short Title of Project:

Effects of Nicotine on Free Acetylcholine in the Hippocampus During Learning

4. Proposed Starting Date: June 1, 1973 (Anniversary of original grant)

5. Anticipated Duration of this Specific Study: One Year

6. Brief Description of Objectives or Specific Aims:

There are no changes in the objectives or specific aims of the project. However, the applicant is confident of receiving a Special Fellowship from the National Institute of Mental Health for a 15-month study at Karolinska Institute in Stockholm beginning May 16, 1973. Although the fellowship has been sought for the purpose of studying a new chemical method for application to brain neurotransmitter analysis ("mass fragmentography") during ethanol intoxication, it is apparent that the fellowship will offer a unique opportunity to study brain neurotransmitters during nicotine's action, as is the intent of the present CTR-USA project. The proposed extension of the present project is outlined in 8 below.

An extension of the present project would be unwarranted if it were not for the progress made during the first 7 months of the project. This progress is detailed in the accompanying progress report, but briefly, these are the accomplishments:

- A. Discovery, through a large number of push-pull cannula experiments and leech muscle bioassays, that nicotine releases free acetylcholine from the rabbit hippocampus in a dose-response manner.
- B. Discovery that large doses of mecamylamine, a central nicotinic antagonist, blocks the learning-facilitation action of nicotine, whereas small doses do not.
- C. Discovery that nicotine, so far, does not produce a marked hippocampal theta rhythm in telemetry-monitored EEG, in doses which facilitate learning.

(continued on page 1a)

7. Give a Brief Statement of your Working Hypothesis:

Nicotine enhances learning by increasing the release of free acetylcholine in the hippocampus, that part of the brain which reputedly mediates learning.

(No change from previous hypothesis.)

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6. Objectives or Specific Aims (Continued):

- D. Rat-size push-pull cannulas have been obtained and are partially working, although we have no concrete data as yet on nicotine's effect on acetylcholine release in rats.

Thus, the present project as outlined in the earlier application is more than halfway completed. The results indicate that further studies on nicotine's effects on neurotransmitter release are warranted. It will be the objective of the extended project to utilize the equipment at Karolinska Institute to:

- A. study the release of hippocampal norepinephrine in relation to hippocampal acetylcholine, in order to gain further insight into the specificity or non-specificity of nicotine in altering neurotransmitters, and
- B. utilize the computerized behavioral system available at Karolinska to study apparent discrepancies in our atropine-nicotine data (see progress report for a discussion of the discrepancies).

(Details of the methods for carrying out the above objectives are given in 8 below.)

In support of the decision to look at hippocampal levels of norepinephrine, Hall and Turner (1972) have recently shown that nicotine injected intravenously in cats causes an increased release of norepinephrine from the hypothalamus. The question thus arises as to whether nicotine does the same thing in the hippocampus and whether such changes can be correlated with nicotine's enhancement of learning. Schechter and Rosecrans (1972) have proposed an interesting hypothesis that states that nicotine may act on a specific nicotine-sensitive cholinergic receptor in the CNS, which causes release of norepinephrine, along the lines of the Burn and Rand hypothesis. Such an hypothesis is extremely important in deciding the pharmacologic mechanism of nicotine's central effect. Looking at both acetylcholine and norepinephrine release from the hippocampus in the proposed experiments should provide interesting data to test Schechter and Rosecran's hypothesis.

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8. Details of Experimental Design and Procedures: (Attach Separate Pages)

The following experiments will be performed at Karolinska Institute (see Physical Facilities Available below for useable equipment):

A. Rabbit experiments - Large albino rabbits will be prepared in the same manner as in the original application (unanesthetized, immobilized with a neuromuscular blocker, and implanted with a push-pull cannula into the hippocampus). Perfusates, collected as described earlier, will be assayed for norepinephrine in addition to acetylcholine. The techniques for analyzing both of these chemicals by the "mass fragmentography" method are to be learned in Stockholm (see attached application for Special Fellowship). Acetylcholine and norepinephrine have been analyzed by this method in Holmstedt's laboratory (Hammar et al., 1968) and other laboratories (Koslow et al., 1972). Acetylcholine itself will first be studied by using the pyrolysis-gas chromatography method which is readily available in Holmstedt's laboratory, and later it will be studied using the more sophisticated mass fragmentography method, after it has been perfected by the applicant.

B. Rat experiments - The computerized behavioral equipment at Karolinska will provide an opportunity to study the problems we have been having with failure to

(see page 2a)

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

(see page 2a)

10. Additional Requirements:

The items listed in the budget on page 3 can be justified as follows:

- A. The full-time research technician is needed to work on the nicotine project because 100% of the applicant's time must be spent on satisfying the Special Fellowship objective, which is to learn the mass fragmentography technique and apply it to the identification of neurotransmitters during ethanol intoxication.
- B. Supplies are required to perform the nicotine experiments. Although the Special Fellowship provides some supply money, this money can not be used to carry on the nicotine project.
- C. The $\frac{1}{2}$ -time secretary, at the University of Kansas, would be extremely valuable in forwarding mail, typing one or two planned manuscripts on our present nicotine work, and keeping an account of the nicotine project supply funds. The department plans to obtain a temporary replacement for the applicant, and secretarial help in the department is not sufficient to take care of the applicant's needs as well as the replacement's needs.

11. Biographical sketches of all principal and professional personnel (append)

There have been no significant changes in the biographical sketch of the principal investigator.

(see page 2b)

12. List of publications: (Five most recent as pertinent) (append)

(see page 2b)

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8. Details of Experimental Design and Procedures (Continued):

replicate our earlier observations that atropine blocks nicotine's enhancement of avoidance learning. Rats will be trained on the same discriminated lever-press avoidance schedule as we have been using, and the characteristics of their learning speed and performance will be analyzed by the on-line PDP-12 computer in an attempt to determine whether earlier atropine blockade of nicotine's effect was an artifact or a real pharmacologic action.

The computer has the advantage that it can show moment-to-moment performance of the animal so that we can observe whether nicotine has a short duration of action followed by a rebound (opposite) effect within an hourly training session. Generally, the behavior of the rat can also be "dissected" by the computer into individual components to provide further information on the effects of nicotine and atropine on the behavior.

References

- Hall, G.H. and Turner, D.M., "Effects of nicotine on the release of ^3H -nor-adrenaline from the hypothalamus", *Biochem. Pharmacol.* 21: 1829-38 (1972).
- Hammar, C.-G., Hanin, I., Holmstedt, B., Kitz, R.J., Jenden, D.J., and Karlen, B., "Identification of acetylcholine in fresh rat brain by combined gas chromatography-mass spectrometry", *Nature* 220: 915-17 (1968).
- Hammar, C.-G., Holmstedt, B., Lindgren, J.-E., and Tham, R., "The combination of gas chromatography and mass spectrometry in the identification of drugs and metabolites", *Adv. Pharmacol. Chemoth.* 7: 53-89 (1969).
- Koslow, S.H., Cattaberrri, F., and Costa, E., "Norepinephrine and dopamine: assay by mass fragmentography in the picomole range", *Science* 176: 177-80 (1972).
- Schechter, M.D. and Rosecrans, J.A., "Nicotine as a discriminative stimulus in rats depleted of norepinephrine or 5-hydroxytryptamine", *Psychopharm.* 24: 417-29 (1972).
- Schmidt, D.E., Szilagy, P.I.A., Alkon, D.L., and Green, J.P., "Acetylcholine: release from neural tissue and identification by pyrolysis-gas chromatography", *Science* 165: 1370-71 (1969).

9. Physical Facilities Available (Continued):

The following facilities, which will be available for my use, are located in the Department of Toxicology, Karolinska Institutet, Stockholm, Sweden and are under the direction of Professor Bo Holmstedt, Chairman of the department:

- A. An LKB 9000 gas chromatograph-mass spectrometer ("mass fragmentographer") which is a sensitive instrument used for detecting small quantities of specific chemicals released from biological tissue (Hammar et al., 1969).
- B. Two Series 1200 Varian gas chromatographs with pyrolyzers for analysis of small quantities of acetylcholine (Schmidt et al., 1969).
- C. Two behavioral test chambers, with a PDP-12 computer for analysis of lever-press behavior inside the chambers.

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D. Office space and desk.

E. A surgical area for push-pull cannula implantations.

11. Biographical sketches of all principal and professional personnel (Continued):

* The research technician who will work on the project in Sweden has not yet been chosen. The only technique that we are now using that will be needed in Sweden is the rabbit push-pull perfusion method. This is capable of being learned by a competent research technician in one month. There is a chance that the technician now working on the project will be able to go to Sweden, which would obviously be advantageous to the project.

12. List of publications:

1. Erickson, C.K., Thorn, S. and Miller, T., "Studies on the mechanism of avoidance facilitation by nicotine", Abstracts of 116th Annual Meeting of the Amer. Pharm. Assoc., Montreal, Canada, pg. 105 (May, 1969).
2. Erickson, C.K. and Patel, J., "Facilitation of avoidance learning by post-trial hippocampal electrical stimulation", J. Comp. Physiol. Psych. 68: 400-6 (1969).
3. Erickson, C.K. and Burnam, W.L., "Cholinergic alteration of ethanol-induced sleep and death in mice", Agents and Actions 2: 8-13 (1971).
- * 4. Erickson, C.K., "Studies on the mechanism of avoidance facilitation by nicotine", Psychopharmacologia 22: 357-68 (1971).
5. Erickson, C.K., "Facilitation of discriminated leverpress avoidance learning by ribaminol", Life Sci. 11: 23-32 (1972).
- ** 6. Erickson, C.K. and Graham, D.T., "Alteration of cortical and reticular-acetylcholine release by ethanol in vivo", J. Pharmacol. Exp. Therap. (In Press, 1973).

* Copies recently sent to the Council.

** Copy submitted with this application.

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13. Budget: (1st year)

(The grant will be administered by the University of Kansas, which will transfer funds for the items marked with * to Karolinska Institute as needed. The Business Officer whose signature appears below has approved this plan for the University.)

NOTE:

A. Salaries (Personnel by names)

% time

Amount

Professional

Technical

*Research technician (12 months, including fringe benefits)

100%

\$8,200

Secretarial help, to forward mail, type manuscripts, etc. (\$2/hour)

10 hr/week

1,040

Sub-Total

\$9,240

B. Consumable Supplies (list by categories)

*Gas chromatographic supplies

100

*Rabbits, rats, food, bedding

800

*Drugs and Chemicals

200

Sub-Total

\$1,100

C. Other Expenses (itemize)

None

Sub-Total

D. Permanent Equipment (itemize)

None

E. Overhead (15% of A + B + C)

\$1,551

(15% of \$10,340)

Total

\$11,891

Estimated Future Requirements:

Salaries

Consumable Suppl.

Other Expenses

Permanent Equip.

Overhead

Total

Year 2

Year 3

Signature

Carlton K. Erickson

Director of Project Carlton K. Erickson

(913) 864-4002

Telephone

Signature

Business Office of the Institution Henry L. Snyder

(913) 864-3126

Telephone

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current

Title of Project	Source	Amount	Duration
1. "Effects of nicotine on free acetylcholine in the hippocampus during learning"	CTR-USA	\$10,550	6/1/72 to 5/31/73
2. "Central neurotransmitter effects of ethanol"	National Institute on Alcohol Abuse and Alcoholism (NIMH)	\$30,901	6/1/72 to 5/31/73
3. "Investigation into an animal model for familial dysautonomia"	University of Kansas General Research Fund	\$2,720	7/1/72 to 6/30/73

Pending

1. Special Fellowship - "Analysis of neurotransmitters by mass fragmentography"	National Institute of Mental Health	Salary and supplies (to be determined)	5/16/73 to 8/15/74
2. "Search for a common mechanism of alcohol-antagonizing drugs"	University of Kansas General Research Fund	\$4,100	7/1/73 to 6/30/74
3. "Relationship between alcohol-blocking drugs"	Licensed Beverage Industries, Inc.	\$10,934	6/1/73 to 5/31/74

1003538850

SEVEN-MONTH PROGRESS REPORT

CTR GRANT #629C

PROGRESS REPORT NO. 1

Carlton K. Erickson, Ph.D.
School of Pharmacy
Department of Pharmacology and Toxicology
The University of Kansas
Lawrence, Kansas

June 1, 1972 - Dec. 31, 1972

Effects of Nicotine on Free Acetylcholine in the Hippocampus During Learning

The application for a research grant submitted to CTR-USA on February 7, 1972 for this new project listed two major objectives (with several associated techniques) as follows:

1. To measure the output of acetylcholine (ACh) from the hippocampus (HPC) in restrained rabbits, before and after the administration of learning-enhancement doses of nicotine.
 - a. Perfusion of the HPC with "push-pull cannulas".
 - b. Recording of EEG from the HPC with bipolar electrodes contralateral to the cannula.
 - c. Measurement of blood pressure for monitoring the cardiovascular effects of nicotine.
 - d. Refinement of the leech muscle ACh bioassay.
2. To do similar experiments in rats that are actively learning a conditioned avoidance response.
 - a. Development of small push-pull cannulas to perfuse the HPC of rats.
 - b. Development of leech-muscle assay sensitivity to measure rat-brain quantities of ACh.
 - c. Perfusion of HPC during behavioral performance (i.e. avoidance learning) in rats.
 - d. Correlation of HPC ACh output with nicotine-enhanced avoidance learning.

During the first half of the project, we have collected a very large amount of valuable data. Not only is the above outline about 5/8 completed, but other related behavioral and telemetry experiments with nicotine have proven extremely fruitful. The reasons for this success are many: a) the experiments have run smoothly because most of the techniques had already been developed for another project, b) two undergraduate students were available to help with collection of data, and c) most experiments gave positive results without the procedural problems which had impeded us in our last nicotine projects.

Results of experiments which have been completed or are in progress are as follows:

1. Analysis of free ACh in the rabbit HPC (almost complete) - A total of 20 rabbits was used to obtain HPC perfusates before and after

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nicotine. Contralateral EEG and femoral artery blood pressure were monitored with a Grass Model 7 polygraph. Nicotine was given in intravenous doses of 0.2, 0.4, and 0.6 mg/kg, which are roughly equivalent to intraperitoneal avoidance-facilitating doses in rats. The time-sequence effects of each dose of nicotine on ACh levels in 15-minute perfusate samples from the HPC are shown in Figures 1, 2, and 3. As can be seen, 0.2 mg/kg nicotine had very little effect on the release of ACh from the HPC, 0.4 mg/kg had a significant effect, and 0.6 mg/kg had a large effect. Furthermore, when a second dose (0.4 mg/kg) of nicotine followed the initial dose (Figure 2), there was an even greater effect on ACh release, perhaps due to cumulative action of the two doses. Interestingly, the effects of the second dose were more fleeting than the effects of the first dose, a suggestion that ACh stores were being depleted by nicotine. The implications of such observations are that nicotine is apparently releasing "free" ACh in the HPC at a rate faster than it can be synthesized, and this effect, if it is occurring in the rat during nicotine-enhanced avoidance learning, means that the excess free ACh could be enhancing the activity of the HPC to cause a direct increase in learning speed. It is probable that there is an optimal level of ACh required for enhanced learning, and that levels that are too high (or too low, if ACh becomes too depleted) would not affect learning. Data aimed at looking further into this mechanism will be collected in the rat experiments.

Figure 4 shows the true dose response nature of nicotine's effect on free ACh. Figure 5 shows that these doses produce theta rhythm, which is characteristic of cholinergic drug effects in the HPC, in some rabbits. Blood pressure from these doses of nicotine is not greatly affected, although in some cases the drug may produce a slight depression of blood pressure. Figures 6 and 7 are cross-sections of the rabbit brain showing the position of the cannula and electrode.

2. Analysis of free ACh in the rat HPC during learning (over 1/2 complete) - Push-pull cannulas have been obtained from Plastic Products Company (Roanoke, Virginia) and have been unilaterally implanted into the HPC of approximately 15 rats. Perfusates have been collected intermittently, with the major problem being clotting of the "pull" side of the cannula (with a subsequent increase in intracranial pressure and death). When perfusates have been successfully collected, they have not been assayed, since the animals up to this point have been anesthetized, and it is known that anesthetics reduce ACh levels to almost imperceptible levels. The important finding, however, is that the quantities of perfusate appear to be great enough to be measured by the leech bioassay technique. A few trials have been run in which blocked cannulas were used while the rat was awake, to find out how the two polyethylene tubes would react to constant turning and twisting by the animal. It appears that this will not present much of a problem. We are continuing to work on the clotting problem of the push-pull cannula, having consulted with Dr. Larry Stein of Wyeth Laboratories, who has published on this technique.

In addition to the above major portions of the proposed project, the following related studies have been done which are extensions of the project funded by CTR-USA last year:

3. Blockade by mecamylamine of nicotine-enhanced avoidance behavior -

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Mecamylamine, a proposed central nicotine antagonist, was injected intraperitoneally into rats 20 minutes prior to nicotine, which was given immediately before every daily one-hour session during discriminated leverpress avoidance training. As can be seen from Figure 8, the larger (1.0 mg/kg) of two doses of mecamylamine completely blocked the avoidance-enhancing effects of nicotine, whereas the smaller dose (0.5 mg/kg) had no effect. Appropriate controls show that the large dose of mecamylamine alone did not affect avoidance behavior compared with saline control. Although the exact central mechanism of mecamylamine is unknown, we have confirmed earlier studies by other workers who showed that mecamylamine does antagonize the central effects of nicotine.

4. Effects of avoidance-enhancing doses of nicotine on telemetry-monitored HPC EEG - In an attempt to correlate nicotine-learning facilitation with changes in HPC EEG, unilateral bipolar stainless steel or platinum electrodes were implanted in the HPC of about 20 rats. A back-pack transmitter was connected to the electrodes when each animal was tested, and the signal sent out by the transmitter was received by a Narco Biosystems Telemetry Receiver, the output of which was amplified and recorded on a Narco Biosystems 4-channel physiograph. Figure 9 shows a typical record of HPC EEG from an awake, moving rat. Although "movement artifacts" were a problem at times, sufficiently interference-free recordings were made to allow EEG monitoring after doses of 0.4-0.6 mg/kg nicotine intraperitoneally. No significant changes in HPC EEG were seen after nicotine. Although previous work in our laboratories has shown that arecoline, a strong cholinergic stimulant, would produce "theta rhythm" (4-7 Hz recordings) in rats with wires connecting the HPC electrodes and the recorder, similar doses of arecoline did not produce theta rhythm in the telemetry-monitored preparation. We are continuing to study the reasons for this by returning to acute, anesthetized rat studies in an attempt to record arecoline-induced theta rhythm with telemetry. After we do this experiment, we will have a better idea about what our problems are.
5. Lack of effect of atropine in blockade of nicotine-enhanced avoidance behavior - In an attempt to replicate and expand upon our earlier work which showed that atropine, a specific "muscarinic" cholinergic blocker, could prevent nicotine's effect on avoidance learning, we again injected 1.0 mg/kg atropine intraperitoneally into rats 20 minutes before nicotine was given at the start of each daily one-hour training session. Providing appropriate controls (saline, atropine alone), and trying three 15-session replications, we were unable to show, as we have before, that atropine had any blocking effect (see Figure 10). There are a number of possible explanations for this, including seasonal variation, different ages of animals, inability to pick out atropine's effect within each hour session (whereas earlier atropine had a longer duration and affected the whole hour's avoidance), animal supplier variation, etc. We would like to continue to look at this problem, using a large group of new rats, all from the same supplier within a short time span, and with behavioral equipment that will allow us to break down the behavior of the animals within each hour. The answer to this problem may provide an important clue to nicotine's central mechanism.

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Summary

A large quantity of valuable information has been collected during the first 7 months of the study. Of greatest importance is the discovery that nicotine causes the release of free ACh from the HPC in a dose-response manner, thereby proving part of the proposed hypothesis. Other important information is the discovery that mecamylamine, a central nicotine antagonist, can prevent the learning-enhancing effects of nicotine. Of potential importance is the finding that atropine, unlike its effects in our earlier work, will not antagonize nicotine-learning enhancement at this time. The reasons for this must be determined. Finally, important developmental work has been done on a) the methods of telemetry-monitored HPC EEG and push-pull cannula perfusion of the HPC in freely-moving, awake rats, and b) their application to the study of nicotine's effects on learning.

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Fig. 1. Effect of 0.2 mg/kg nicotine i.v. on acetylcholine release from the rabbit hippocampus.

C = 15 MINUTE PERIODS BEFORE NICOTINE

N = 15 MINUTE PERIODS AFTER NICOTINE

() = # OF REPLICATIONS

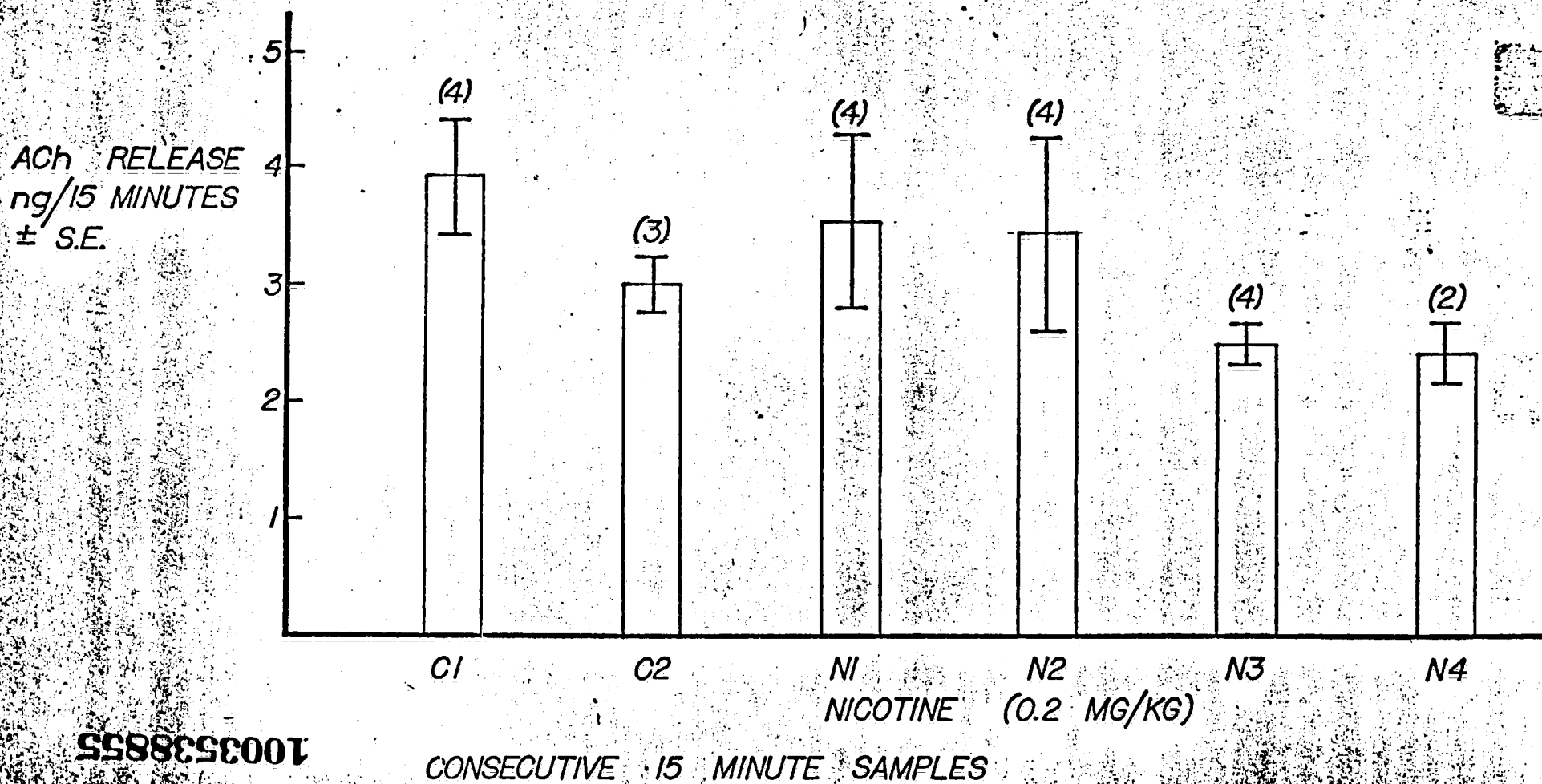


Fig. 2. Effect of 0.4 mg/kg nicotine i.v. on acetylcholine release from the rabbit hippocampus. The same dose of nicotine was given again at the second arrow.

C = 15 MINUTE PERIODS BEFORE NICOTINE
N = 15 MINUTE PERIODS AFTER NICOTINE
A = SECOND INJECTION OF NICOTINE

() = # OF REPLICATIONS

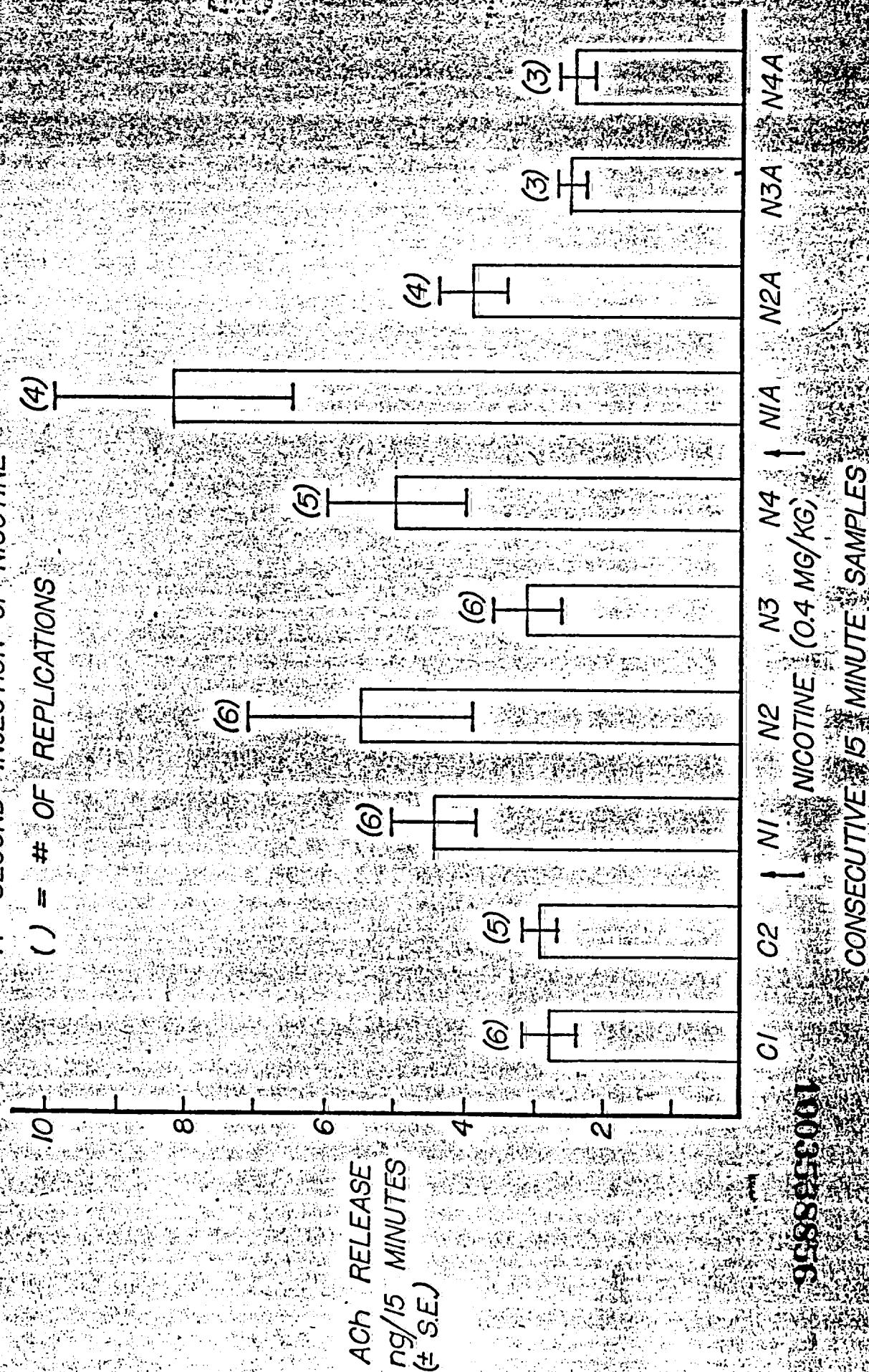
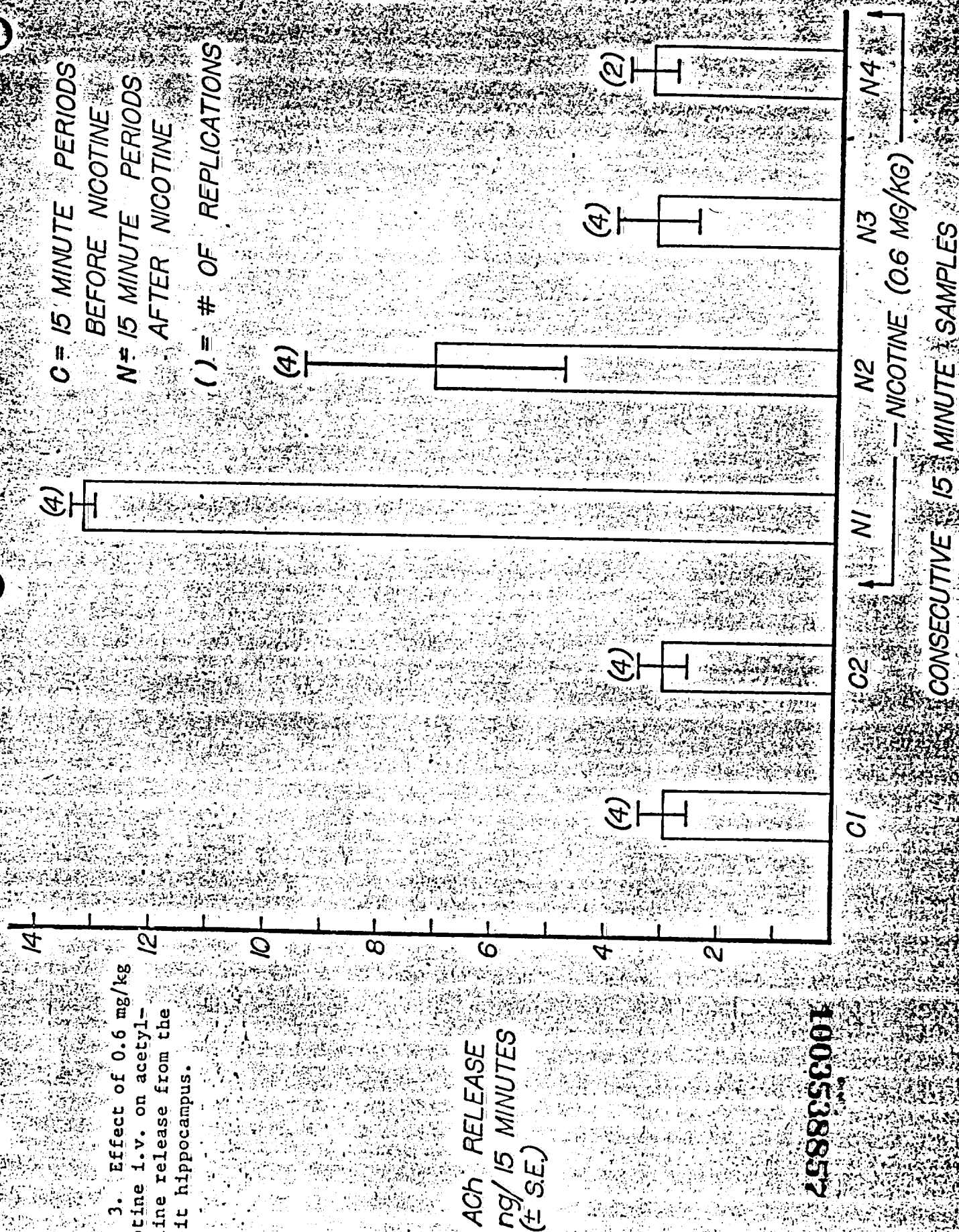
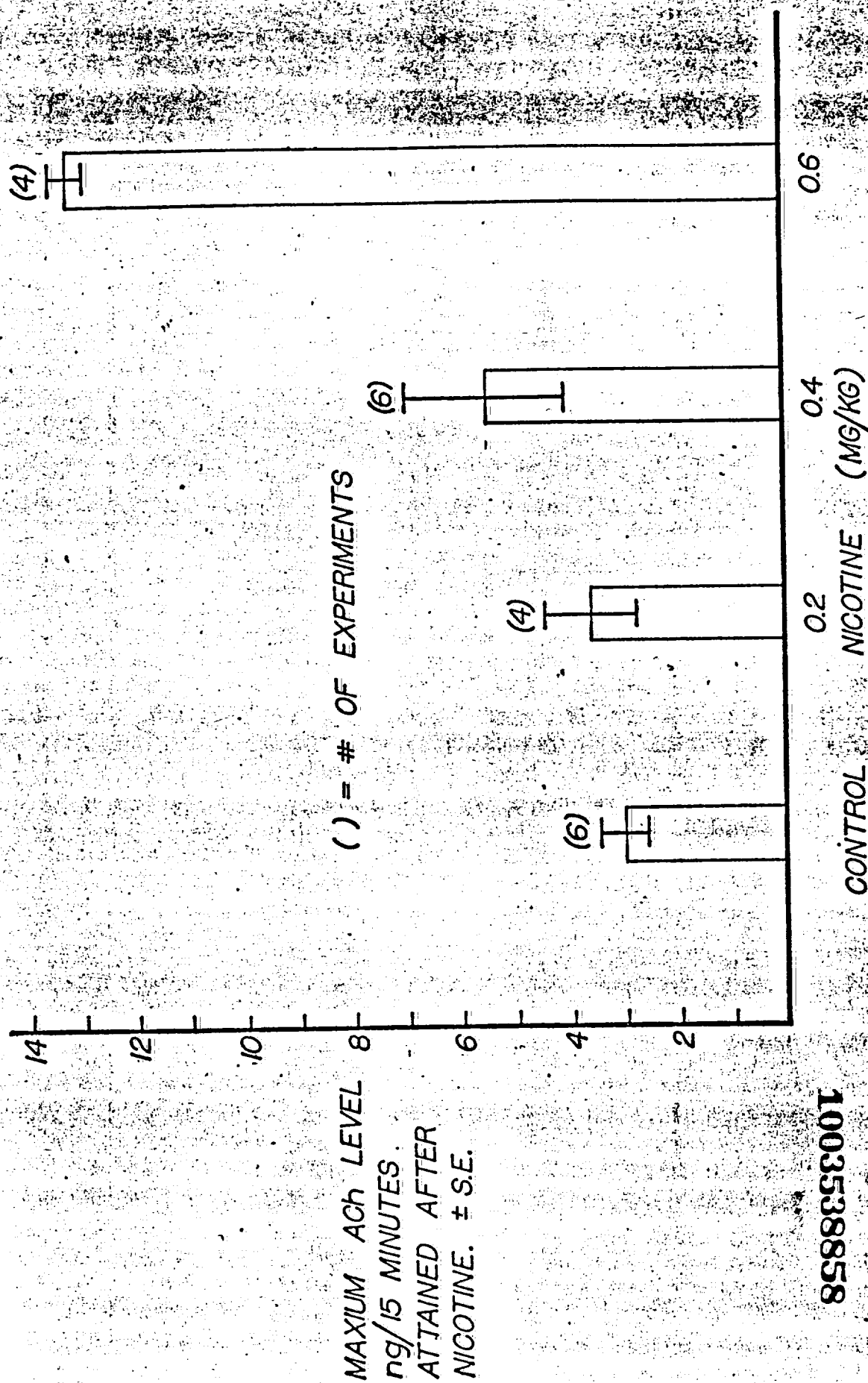


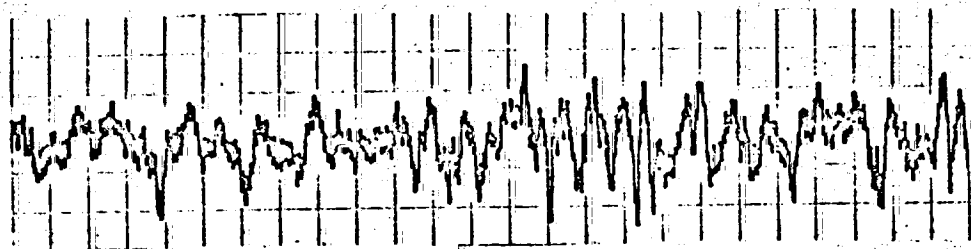
Fig. 3. Effect of 0.6 mg/kg nicotine i.v. on acetylcholine release from the rabbit hippocampus.



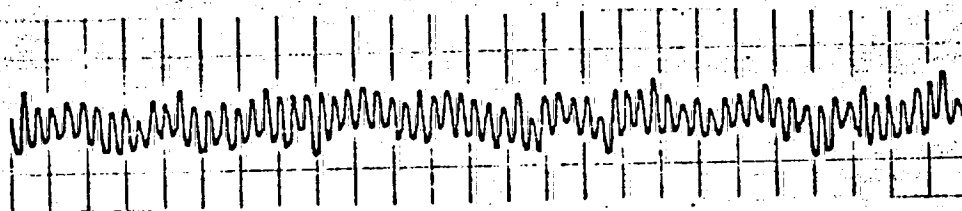
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Fig. 4. Maximum acetylcholine release from the rabbit hippocampus following the three doses of nicotine, and during control (no nicotine) determinations.





A. 1 sec



B. 1 sec

Fig. 5. Representative hippocampal electrical patterns in an unanesthetized, immobilized (gallamine) rabbit before (A) and 20 minutes after (B) an intravenous dose of 0.4 mg/kg nicotine. Calibration sensitivity for both records is the same. Activity in B is theta rhythm, 4-7 H₂.

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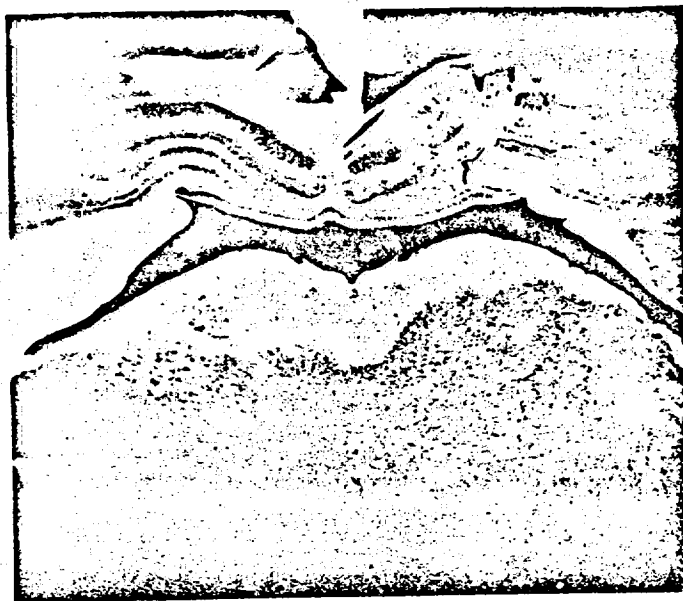


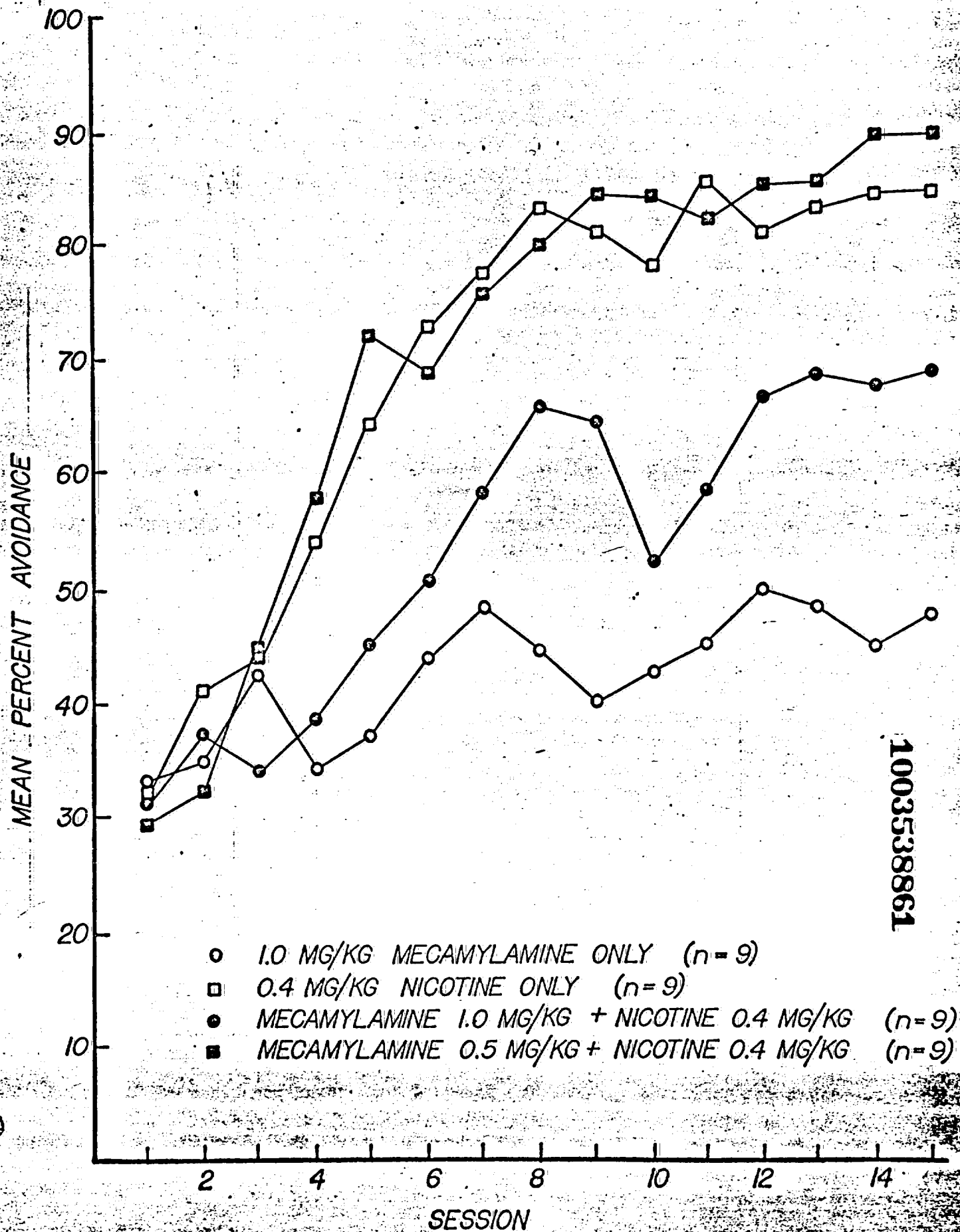
Fig. 6. Cross section of rabbit brain, showing push-pull cannula placement in the dorsal hippocampus.



Fig. 7. Cross section of rabbit brain, showing bipolar electrode placement in dorsal hippocampus, contralateral to and approximately 0.5 mm rostral to cannula tract in Fig. 6.

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Fig. 8. Blockade of nicotine-induced conditioned avoidance learning enhancement by two doses of mecamlamine given intraperitoneally.



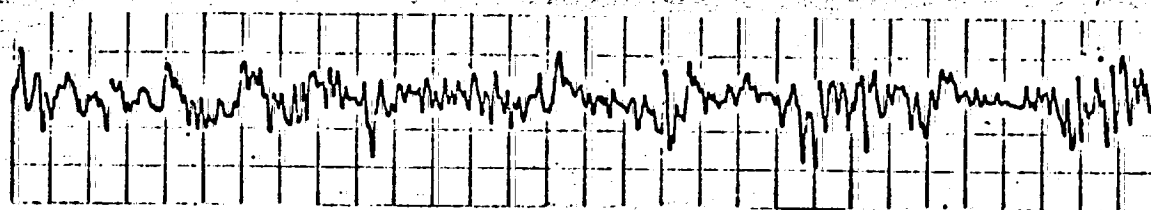
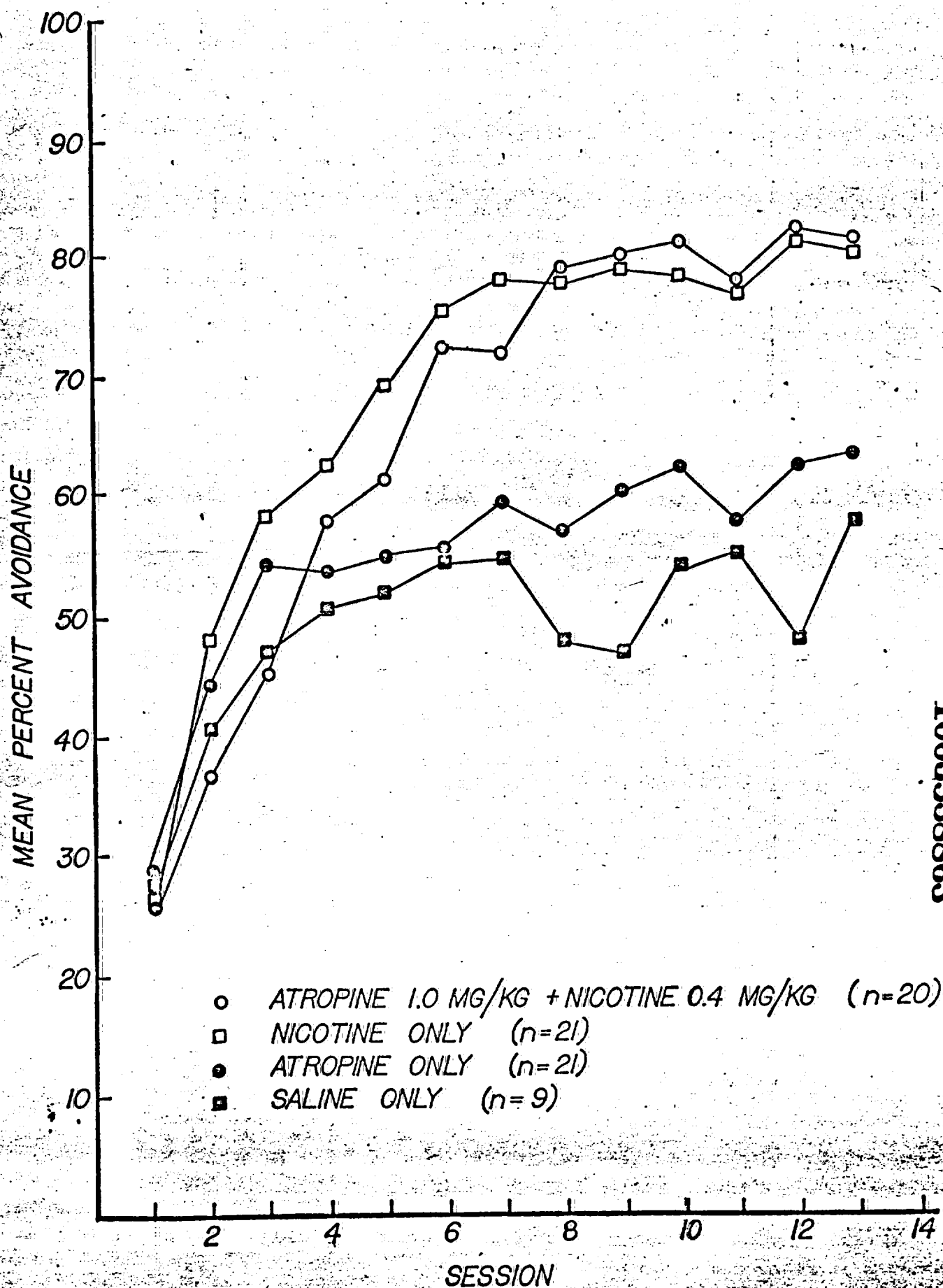


Fig. 9. Representative hippocampal electrical activity through platinum electrodes via telemetry in a rat (uncalibrated amplitude).

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Fig. 10. Lack of blockade of nicotine-induced conditioned avoidance learning enhancement by atropine given intraperitoneally.



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#042B GOLDSTEIN

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THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

November 28, 1972

2.

Grant application No. 642B

To: The committee comprising Drs. Bing, Cattell, and Meier

Subject: Leonide Goldstein, D.Sc., N. J. College of Medicine and
Dentistry, New Brunswick, N. J.
Continuation application No. 642B
"Behavioral and Electrophysiological Effects of the
"Chronic Nicotine State" in Rats"

History

Grant No. 642A, 1971, \$20,240

Grant No. 642AR1, 1972, \$23,230

Application 642B requests \$28,579 for 1973, plus one additional year.

Documents submitted (attached)

1. Application dated October 20, 1972
2. C. V. of Dr. Goldstein
3. C. V. of Dr. Nelsen

Comment

The applicant moved to Rutgers November 1, 1972, as a result of the closing of his previous institution. He visited the CTR office September 26, 1972, and described his new facilities and setting as satisfactory. Key equipment purchased with CTR funds can be transferred if activation of his support at Rutgers is approved.

As support is requested starting January 1, 1973, the Planning Committee will be asked to take interim action on December 8, 1972. An alternative would be to allow the use of CTR funds unexpended at December 31, 1972 to pay Dr. J. M. Nelsen until the full SAB can act in March 1973.

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F.W.N.

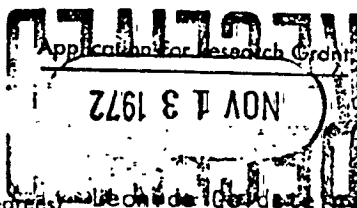
FWN:wg
Encls.
cc: Planning Committee

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Oct. 11/13/72

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022



Date: October 20, 1972

1. Name of Investigator(s): (include Title and Degrees) Leonard B. Glaser, M.D. Sc. Associate Professor of Psychiatry. NJ College of Medicine and Dentistry. New Brunswick, N.J. Judith M. Nelsen, Ph. D. Post-Doctoral Fellow, same address.
2. Institution & Institute for Mental Health Sciences. CMDNJ. Rutgers Medical School.
Address: P.O. Box 101. Piscataway, N.J. 08854.

3. Short Title of Project: Behavioral and electrophysiological effects of the "chronic nicotine state" in rats.

4. Proposed Starting Date: January 1, 1973

5. Anticipated Duration of this Specific Study: 2 years

6. Brief Description of Objectives or Specific Aims: See attached pages.

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7. Give a Brief Statement of your Working Hypothesis: Chronic nicotine treatment produces changes in the balance between reticular formation and limbic system arousal, enhancing "motivational" arousal and reducing "drive arousal".

8. Details of Experimental Design and Procedures: (Attach Separate Pages)

See attached pages

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location) The Laboratory at the Institute for Mental Health Sciences is comprized of 2 rooms, one 10'x20" and one 20'20'. We will have available all the equipment presently at the N.J. Neuropsychiatric Institute, including the items bought on the funds provided by the Council. A complete set up for animal care & maintenance will be provided.

10. Additional Requirements:

The only additional requirement will be for the purchase of a constant current stimulator needed for the experiments in which the RF will be directly stimulated.

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11. Biographical sketches of all principal and professional personnel (append)

See attached lists

12. List of publications: (Five most recent as pertinent) (append)

See attached list

3.

13. Budget (1st year)

A. Salaries (Personnel by names) (16.66% Fringe benefits)

% time

Amount

Professional

Judith M. Nelsen, Ph. D.

100

R

Leonide Goldstein, D. Sc.

10

Technical

Katheleen Pelley, B.A.

100

R

Sub-Total

R

B. Consumable Supplies (list by categories)

Animals

200.00

Food

200.00

Maintenance

100.00

Drugs, chemicals, EEG paper, electrodes

200.00

Sub-Total

700.00

C. Other Expenses (itemize)

Travel. Principal investigator. 2 Meetings

400.00

Publications. Reprints

250.00

Sub-Total

650.00

D. Permanent Equipment (itemize)

Constant voltage stimulator. Model 7151

Nuclear Chicago

1,000.00

1,000.00

E. Overhead (15% of A+B+C)

Total

3,597.30

28,579.30

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	R	500.00	600.00		3,726	28,726
Year 3						

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature Leonide Goldstein, D. Sc.

Director of Project

Signature W. H. Chapin

Business Officer of the Institution

Telephone

Telephone

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Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current

Title of Project	Source	Amount	Duration
Effects of microwave radiations on the EEG	US Navy Air-Development Center Warminster, Pa	25,000.00	1 year
Effects of a Prostaglandin analog on the EEG and behavior	Office of Naval Research. Arlington Va.	20,000.00	1 year

Pending

Effects of a Prostaglandin analog on the EEG and behavior	Office of Naval Research.	20,000.00	1 year
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We reported previously, on the basis of studies carried under Grants from the Council for Tobacco Research-USA, that following chronic nicotine treatment there occurs in rabbits changes in certain features of the electrophysiological mechanisms of arousal. These changes have been interpreted as indicating a shift from the "classical" arousal mechanism (involving chiefly the mesencephalic reticular formation (RF)) to a qualitatively different arousal mediated by the limbic system. A proposed consequence of such an electrophysiological change was that behavior should become more specifically goal-oriented since, as Routtenberg pointed out (Psychol. Rev. 75, 51-80, 1968), the main feature of limbic system arousal is "incentive-oriented" while that of the RF is "drive oriented".

Working under subsequent Grants from the Council, we tested this proposal in an attention task in rats. We found that indeed chronic nicotine treatment did improve the efficiency of responses to goal-oriented stimuli without causing or be accompanied by a general increase in the behavioral levels of activity.

The results obtained in these studies invite further research directed both towards a better understanding of the mechanisms involved in arousal and of the motivation accounting for the widespread self-administration of nicotine by humans.

If as Routtenberg has suggested (and our present results imply) there exists a balance between the limbic and RF influences on arousal, it would follow that modification of the relationships toward greater limbic system control (resulting from chronic nicotine treatment) should alter the sensitivity of the RF arousal pathway to manipulation. This hypothesis can be tested by direct electrophysiological and pharmacological means. It is known that electrical stimulation of the RF produces cortical activation and that the current needed to elicit the response varies with the state of the brain. We propose to study the threshold for and duration of cortical activation in chronically saline-treated and nicotine-treated rats. It is also known that a number of stimulant and psychoactive drugs exert their effects by acting primarily at the level of the RF. One would expect a comparable change in the efficacy of such drugs following chronic nicotine treatment. The drugs of particular interest would be: D-amphetamine, methyl-phenidate, physostigmine (following protection of the periphery with methyl-atropine), LSD-25, and, in view of the present controversy concerning classification of the nature of the effect, delta-9 THC.

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Besides the study of direct electrophysiological effects at cortical and subcortical levels, we intend to conduct parallel behavioral studies. These would be focused on the elucidation of the functional consequences of the electrophysiological and pharmacological manipulations. The Continuous Attention Task appears well-suited for such studies since it has been demonstrated to be sensitive to both the level and qualitative nature of arousal.

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Item 8.

Two related lines of investigation are planned. The first concerns electroencephalographic measures of cortical activation induced by electrical stimulation via the mesencephalic reticular formation (RF) arousal pathway and by pharmacological agents. In previous applications for "Research Grants" from the Council (dated November 25, 1970 and October 25, 1971), we have reviewed the surgical and EEG measurement technics which we have developed for application to such studies. We propose to prepare with cortical and subcortical (RF) electrodes an experimental group of approximately 20 rats (Holtzman, Sprague-Dawley, male). These animals will undergo adaptation training in an EEG recording chamber. They will then be assigned in random fashion to two groups, one of which will receive chronic treatment with nicotine (100 ug/kg, s.c., t.i.d.) and the other, with physiological saline.

A schedule for delivery of small doses of electrical stimulation to the RF will be carried out to determine the threshold for and the duration of cortical activation resulting from direct stimulation of the RF arousal pathway under conditions of chronic nicotine and chronic saline treatment. Arousal (desynchronization) will be measured and quantified from the cortical EEG recordings. Further, comparisons between the cortical effects of D-amphetamine, L.S.D., physostigmine, and tetrahydrocannabinol in animals treated chronically with either nicotine or saline will be made.

Electrical current will be delivered by a Nuclear-Chicago constant current stimulator. Parameters of the current will be within a moderate range which has been shown to cause only reversible effects both on behavior

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and neural tissue (Kornetsky and Eliasson, 1969; Nelsen, 1970).

The second area of investigation involves behavioral measures of the effects of modifications in the proposed balance between limbic and RF control of arousal. Because in our hands it has proven to be impressively sensitive to levels of arousal, the same form of the behavioral task of Kornetsky and Eliasson (1969) which we have described in previous grant applications to the Council (dated November 25, 1970 and October 25, 1971) will be used to assess the functional consequences of electrical and pharmacological manipulations of the RF arousal pathway.

Twelve rats will be prepared surgically with electrodes at the sensory-motor cortex and in the mesencephalic RF. Following recovery (about three weeks), these animals will be trained to perform on the visual attention task. The rats will be partially food deprived and maintained at approximately 85% of their normal, free-feeding body weights. They will be trained to press a lever for a food pellet reinforcement following the presentation of a conditional stimulus (C.S.) which is the white cue light in a standard operant conditioning box. Training is carried out in a series of phases such that initially the task is quite elementary, i.e., continuous reinforcement or fixed ratio 1 in the presence of a constant C.S. During successive training sessions, an inter-trial interval (I.T.I.) is introduced and the duration of the C.S. is reduced in step-wise fashion while a punishment contingency for inappropriate responding is also added. In the task's final form, the duration of the cue light is only 0.2 sec, followed by an available response time of 5.0 sec during which only the first lever press

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is reinforced. Failure to press after a C.S. is scored as an omission error (o.e.) and has no consequence for the animal other than the loss of a reinforcement pellet. The I.T.I. is variable (so that the rat is not learning to time responses according to a fixed interval) with a mean of 10.0sec. A lever press during the I.T.I. is scored as a commission error (c.e.) and is punished by the imposition of a 30.0 sec "time-out". Additional responses during the time-out reset the punishment clock to 30.0 sec and are also scored as c.e.'s. A session is terminated after 100 reinforcements have been delivered or after one hour has elapsed. The task is programmed via electro-mechanical modules.

This type of task in which the animal is asked not only to make appropriate responses but also to inhibit inappropriate responses is difficult for rats to learn and requires several months of training to achieve efficient performance which we have defined as: 1) for o.e.'s, $\#o.e./\#$ reinforcements times 100 \leq 30%, and 2) for c.e.'s, $\#c.e./\#$ reinforcements times 100 \leq 30%. After the rats have reached criterion levels of performance, half the group will be subjected to chronic treatment with nicotine (100 ug/kg, s.c., t.i.d.) and half to physiological saline. After approximately one week of regular injections, a schedule of low current level electrical stimulation to the RF will be introduced during the testing sessions. Such stimulation has been shown to disrupt performance in a similar task (Kornetsky and Eliasson, 1969) where electric foot-shock was the reinforcing agent. Since based on our previous work, differential baseline behavior is expected depending on whether rats are nicotine- or saline-treated, each group (in

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fact, each animal) will act as its own control. Our hypothesis is that because nicotine-treated rats are in a state of greater incentive-oriented arousal (hippocampal predominance in the control of cortical function), their performance will be affected less detrimentally by RF stimulation than the performance of saline-treated animals.

Further, behavioral studies are planned which will focus on the possible protective action of the chronic nicotine state on the disruptive effects of pharmacological agents known or suspected to act on the RF arousal pathway by causing a relative increase in general or drive-oriented arousal and hence, decrease in incentive-oriented arousal. These agents will include D-amphetamine, L.S.D., physostigmine, and tetrahydrocannabinol. They will be administered according to a randomized block design and the results will be tested by applying an analysis of variance.

Kornetsky, Conan and Eliasson, Mona: Reticular Stimulation and Chlorpromazine: An Animal Model for Schizophrenic Overarousal. Science 165: 1273-1274, 1969.

Nelsen, Judith M.: Single Dose Tolerance to Morphine Sulfate: Electroencephalographic Correlates in Central Motivational Systems. Unpublished Doctoral Dissertation. (Boston University) 1970.

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Bhattacharya, I.C. and Goldstein, L. Influence of acute and chronic nicotine administration on intra- and inter-structural relationships of the electrical activity in the rabbit brain. *Neuropharmacol.* 8, 109-118, 1970.

Greenberg, R.S. and Goldstein, L. An EEG study of the relationships between brain structures in rabbits under ethanol and d-amphetamine. *Quart. J. Studies Alcohol.* 30, 843-848, 1969.

Goldstein, L., and Dolge, G. An analysis of the effects of ethanol on cortical and subcortical electrical activity in cats and rabbits. *in: Interm. Symposium Biological Aspects of Alcohol Consumption.* O. Forsander and R. Erikson, ed. Finnish Foundation for Alcohol Studies, 20, 267-274, 1972.

Nelsen, J.M., and Goldstein, L. Improvement of performance on an attention task with chronic nicotine treatment in rats. *Psychopharmacologia*, 26, 347-360, 1972.

Nelsen, J.M., and Goldstein, L. Chronic nicotine treatment: acquisition and performance of an attention task by rats. Submitted for publication: *Neuropharmacology*, 1972.

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Curriculum VitaeLeonide Goldstein, D.Sc.

Born:

R

Permanent Residence in the United States - Alien registration No. REDACTED

Marital Status:

REDACTED

REDACTED

Student at the Conservatoire National de Musique, Paris

Graduated in violin, harmony and composition.

Military duty, French Army

Technical Assistant Institut de Biologie, Paris

Research Assistant

Non-commissioned Officer, French Army

Undergraduate studies University of Paris

Research Associate Laboratoire de Physiologie School of Medicine,

University of Montpellier, France

Member of the Research Division of the Free French Forces

Special assistant to Dr. H. J. Muller, Amherst College, Amherst, Mass.

B.A. and M.A. Amherst College

1945-47 Assistant Professor University of Paris (Physiology & Genetics)

1947-53 Acting Director Laboratoire de Biometrie of the French National
Research Council

1951 Doctor of Sciences degree. University of Paris, Sorbonne

1953-58 Assistant Professor, Neurophysiology, Ecole Pratique des Hautes
Etudes, Sorbonne

1958-61 Associate Professor, Pharmacology, Emory Univ., Atlanta, Georgia

1951-64 Neuropharmacologist, Bureau of Research, Neuropharmacology
Section, N. J. Neuropsychiatric Institute, Princeton, N. J.1964 Research Scientist Grade 1, Bureau of Research, Neuropharmacology
Section, N. J. Neuropsychiatric Institute, Princeton, N. J.

1966 Consultant - McNeil Laboratories, Fort Washington, Pa.

1969 Visiting Senior Fellow - Department of Biology - Princeton University

1972 Associate Professor of Psychiatry. Rutgers Medical School.

Membership in Scientific Societies:

REDACTED

REDACTED

Honors:Croix de Guerre (1939-40): Medal of the Free French Forces: Palmes Academiques
(1950). Associate Editor "The Journal for the Study of Consciousness" and
"Research Communications in Chemical Pathology and Pharmacology".

President - French Club of Princeton (1969-1971).

Trustee - Schizophrenia Foundation of New Jersey (1972).

Listings:

American Men at Science - Who is Who in the East.

Publications:Author or co-author of 140 papers and abstracts dealing with genetics, endo-
crinology, electro-physiology and pharmacology of the brain.

(Revised February 1972)

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CURRICULUM VITAE

Judith M. Nelsen, Ph.D.

BORN: 2

- Milwaukee, Wisconsin, U.S.A.

MARITAL STATUS:

REDACTED

Primary and secondary studies, public schools of Town of Lake and City of Cudahy, Wisconsin

Undergraduate studies, University of Wisconsin-Milwaukee (Letters and Science, Pharmacy)

Laboratory assistantship in bacteriology (University of Wisconsin-Milwaukee)

Undergraduate studies, University of Wisconsin-Madison (Pharmacy, Psychology)

Research assistantship in physical chemistry (University of Wisconsin-Madison)

Summer research assistantship in physical chemistry (from the U.S. Department of the Army at the University of Wisconsin-Madison)

B.S. (HONORS) degree. University of Wisconsin. Madison, Wisconsin

Graduate studies, Boston University School of Medicine, Division of Medical Sciences, Department of Pharmacology and Experimental Therapeutics (Major professor: Conan Kornetsky, Ph.D., Director, Laboratory of Behavioral Pharmacology)

Graduate School Research Fellowship

1966-70 Public Health Service Research Fellowships (N.I.M.H.)

1970 Doctor of Philosophy degree. Boston University. Boston, Mass.

1970-72 Post-doctoral appointment. N.J. Bureau of Research in Neurology and Psychiatry. Box 1000, Princeton, N.J.

1973 Senior Scientist. Rutgers Medical School. Department of Psychiatry, Piscataway, N.J.

HONORS: Sophomore Honors (U.W.); Senior Honors (U.W.); Sigma Epsilon Sigma (U.W.); Rho Chi (U.W.); Phi Kappa Phi (U.W.); Sigma Xi (B.U.)

PROFESSIONAL SOCIETY MEMBERSHIPS:

REDACTED

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PUBLICATIONS

Nelsen, Judith M. and Conan Kornetsky: Single Dose Tolerance to Morphine Sulfate: EEG Changes. *The Pharmacologist* 10: No.2, 1968.

Weil, Andrew T., Norman E. Zinberg, and Judith M. Nelsen: Clinical and Psychological Effects of Marihuana in Man. *Science* 162: 1234-1242, 1968.

Nelsen, Judith M. and Leonide Goldstein: Improvement of Performance on an Attention Task with Chronic Nicotine Treatment in Rats. *The Pharmacologist* 13: No. 2, 1971.

Nelsen, Judith M. and Leonide Goldstein: Improvement of Performance on an Attention Task with Chronic Nicotine Treatment in Rats. *Psychopharmacologia* 26: 347-360, 1972.

Nelsen, Judith M. and Conan Kornetsky: Morphine-Induced EEG Changes in Central Motivational Systems: Evidence for Single-Dose Tolerance. Fifth International Congress on Pharmacology (Abstracts, p. 166, #993), 1972.

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900 SCHENKER

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 1, 1973

Grant Application No. 900

5
Denied

To: The committee comprising Drs. Bing, Cattell and Meier
Subject: Victor J. Schenker, Ph.D., Temple University, School of Dentistry,
Philadelphia, Pa.
New application No. 900
"Neuroactive Components of Human Saliva and their Possible
Interaction with Nicotine"

History

This project was Case 141 and full application was encouraged.

The request is for \$35,410 plus one additional year.

Documents Submitted (attached)

1. Application dated January 26, 1973 (21 pages, 5 figures).
2. Biographies and bibliographies of the investigators.
3. Reprint "Studies on Human Saliva . . .", Schenker and Schenker, J. Nerv. Ment. Dis. 128 520, 1959.

Comment

Since the classic work in this area of Dr. Vincent F. Lisanti is cited in the application, no outside opinion is being sought.

It is noteworthy that partial salary of the principal investigator is requested.

F.W.N.
F.W.N.

FWN:wg
Encls.

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Comm.

PHARMACOLOGY

#900

Dr. Bing
Dr. Cattell
Dr. Meier

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 50TH STREET
NEW YORK, N. Y. 10022
(212) 421-6385

Application for Research Grant

(Use extra pages as needed)

JAN 31 1973

Date: Jan. 26, 1973

1. Principal Investigator (give title and degrees):

Victor J. Schenker, Ph.D.
Research Professor of Biochemistry

2. Institution & address:

Temple University, School of Dentistry
3223 N. Broad St.
Philadelphia, Pa. 19140

3. Department(s) where research will be done or collaboration provided:

Dept. of Biochemistry, School of Dentistry
Dept. of Pharmacology, School of Pharmacy

4. Short title of study.

Neuroactive Components of Human Saliva and Their Possible Interaction
with Nicotine.

5. Proposed starting date: July 1, 1973

6. Estimated time to complete: 2 years.

7. Brief description of specific research aims: Previous work by this investigator shows the presence of neuroactive material in extracts of human saliva having the spectrofluorometric characteristics of biogenic amines related to tyramine. This is also found in homogenates of human submaxillary gland tissue. Preinjection of extracts containing this salivary fluorophore (SF) in mice caused a 2- to 3- fold prolongation of sleeping time due to hexobarbital. This potentiation does not occur when nicotine is added to SF extracts, suggesting a new approach to the study of the relationship between nicotine and neuroactive biogenic amines. Cholinergic stimulation by urecholine in human subjects elicits a marked increase of SF in saliva, in contrast to atropine which is followed by a decrease indicating autonomic mediation of the elaboration and/or release of SF from the salivary glands. Direct application of SF to the right heart in dogs with heart catheterization elicits marked increase in right ventricular pressure and pulmonary circuit. The proposed research is an attempt to elucidate these findings. Specific aims are directed at the precise chemical identification of SF together with its pharmacological and neurochemical characterization in terms of activity at extra-oral sites (e.g., brain, heart) from possible absorption through the oral mucosa, with particular emphasis upon interaction with nicotine similarly absorbed.

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8. Brief statement of working hypothesis:

2.

An overall working hypothesis stemming from the combined findings of our previous work has been formulated as follows: 1) human saliva contains one or more neuroactive biogenic amines ostensibly elaborated by and/or released from the submaxillary salivary glands under the mediation or modulation of the autonomic nervous system; 2) such compounds, continuously present in the oral cavity, may be absorbed through the oral mucosa and transported directly to the right heart and, via the pulmonary circuit, reach the brain and other organs before chemical alteration by passage through the hepatic or renal circulation. This vascular shunt could thus facilitate the homeostatic interaction of these compounds with other regulatory factors at extra-oral sites. As a corollary, such interaction may be affected by the presence of exogenous agents (e.g., nicotine) in the oral cavity, from which they are absorbed.

9. Details of experimental design and procedures (append extra pages as necessary)

See appended Research Plan, pp.6 through 21

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

see Research Plan - Section 1-D, page 14

11. Additional facilities required:

none

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12. Biographical sketches of investigator(s) and other professional personnel (append): appended ff. page 21

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

see Section 2-C, pp. 20-21

14. First year budget:

A. Salaries (give names or state "to be recruited")

% time

Amount

Professional (give % time of investigator(s)
even if no salary requested)

REDACTED

Dr. Victor J. Schenker (Principal Investigator) 60

Dr. David E. Mann, Jr. (Co-investigator) 10

Dr. Robert L. Pollack (Co-investigator) 10

Technical

Biochemist (to be recruited) 100 7,500

Fringe benefits @12.5% 2,438

Sub-Total for A 21,938

B. Consumable supplies (by major categories)

Chemicals and Glassware 725.00

Chromatographic supplies-resins, plates etc. 625.00

Experimental animals including maintenance 750.00

Spare parts and replacements for SPF 650.00

Duct collection devices 125.00

Sub-Total for B 2,875

C. Other expenses (itemize)

Fees for paid volunteers 200.00

Sub-Total for C 200

Running Total of A + B + C \$ 25,013

D. Permanent equipment (itemize)

1 Aminco-Bowman Model 4-8202
Spectrophotofluorometer complete
with Power Supply, Microphotometer
and Accessories 4,850.00

1 Aminco-Bowman Model 4-8909XY Recorder 1,500.00

1 Aminco-Bowman Time generation device
for above 295.00

Sub-Total for D 6,645

E 3,752

E. Indirect costs (15% of A+B+C)

Total request \$35,410

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	R	\$2,785	\$200	nil	\$3936	\$30,175
Year 3	---					

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5.

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Neurosecretory Components of Human Saliva	Temple University Grant-in-aid, 405-063-75	1,000.00	1 July/72 through 20 June/73

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
	none		

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Victor J. Schenker, Ph.D.Signature *Victor J. Schenker* Date 1/26/73Telephone 215 229-8500 287
Area Code Number Extension

Checks payable to

(Temple Univ. Health Science Center

Mailing address for checks

D.W. Siegel
TUHSC,
3400 N. Broad St. Phila, Penna. 19140

Responsible officer of institution

Typed Name David W. SiegelTitle Assoc. V. P. for AdministrationSignature *David W. Siegel* Date 1/26/73Telephone R
Area Code Number Extension

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1. Research Plan

A. Introduction and Specific Aims

The proposed study represents one facet of this department's total research program in oral biochemistry and physiology. The study is an extension of previous work by the principal investigator which provides presumptive evidence that: 1) human saliva contains material comprising amine-like compounds with neuroactive effects which, in mice, are apparently antagonized by low doses of nicotine; 2) this material is also found in homogenates of submaxillary gland tissues from humans and Rhesus macaque monkeys; and 3) the level of this material in human saliva is apparently mediated by cholinergic activity of the autonomic nervous system. Briefly stated, the relevant experimental data are as follows: Human whole saliva, collected under standardized conditions and extracted by solvent and/or ion-exchange methods specific for phenolic amines, yields material showing the spectrofluorometric characteristics of tyramine-like amines (Figs. 1,2,3). Preliminary separation procedures using thin-layer chromatography with specific dye reagents show the presence of a number of phenolic amine-like constituents some of which show the spectrofluorometric characteristics of tyramine. The pharmacologic action of purified extracts on the aortic spiral strip preparation from rabbits pretreated with iponiazid was the same as that of authentic p-tyramine HCl (3×10^{-5} M) with inhibition by 2×10^{-5} M cocaine. Pilot tests in dogs with heart catheterization showed that small amounts of extract introduced directly into the right heart elicited marked increases in right ventricular pressure and pulmonary circuit. Pretreatment i.v. of mice with small amounts of lyophilized, purified extracts containing microgram amounts of salivary fluorophore (SF) produce a 2- to 3-fold prolongation of hexobarbital induced sleeping time (Table 1) as does a $2 \mu\text{g}$ i.v. dose of authentic p-tyramine HCl. Preliminary tests in mice indicate that such potentiation by SF does not occur when nicotine alkaloid (0.01 mg/kg) is injected together with the extract. Using quantitative spectrofluorometric procedures to measure salivary SF levels in healthy human subjects, it was found that a single small dose (4 mg in 0.8 ml., s.c.) of the cholinergic agent urecholine elicited a marked increase (up to

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6-fold over the saliva control) of SF in saliva within 30 minutes; this increase was not positively correlated with flow volume and subsided to preinjection levels during the subsequent 1-1.5 hours. In contrast, atropine (0.64 mg in 1.6 ml, s.c.) produced the opposite effect; there was a prolonged decrease in SF. Epinephrine (0.3 ml of 10^{-3} adrenaline chloride, s.c.) did not uniformly elicit the marked transitory peak response shown by urecholine; epinephrine showed a prolonged gradual increase in SF parallel to that seen in the saliva control curves, (Figs, 4 and 5).

In an attempt to interpret these combined findings physiologically, an overall working hypothesis has been formulated, thus 1) human saliva contains one or more neuroactive biogenic amines ostensibly elaborated by and/or released from the submaxillary salivary glands under the mediation or modulation of the autonomic nervous system; and 2) such compounds, continuously present in the oral cavity in varying amounts - depending upon autonomic activity - may be absorbed through the oral mucosa and transported directly to the right heart and, via the pulmonary circuit, reach the brain and other organs before chemical alteration by passage through the hepatic or renal circulation. This vascular shunt could thus facilitate the homeostatic interaction of these compounds with other regulatory factors at extra-oral sites. This working hypothesis constitutes a major basis for the present specific aims as well as our long-range goals.

In this context, the preliminary finding that nicotine is an apparent antagonist to SF is of some interest. It is generally recognized that nicotine is absorbed through the oral mucosa, and its pharmacological actions in the central nervous system have been comprehensively reviewed by Silvette, et al.(1). It is therefore intended, in our studies, to place emphasis upon the interactions between SF and nicotine (both pharmacologic and neurochemical) in the CNS. Not only will this exploit this drug as a tool for the pharmacologic characterization of an ostensibly neuroactive substance, but, at the same time, it will disclose possible new information about nicotine mechanisms in the CNS. This is all the more appropriate in the light of the amine-like character of SF and the reported relationship between this agent and catechol amines which stem from the same amino acid precursors as do those contained in SF. (26,27).

With these considerations in mind, our initial specific aims will be directed primarily at the precise chemical identification of the individual components of SF as demonstrated by our chromatographic separation studies. As indicated above,

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pharmacological testing, in collaboration with Professor David E. Mann, Jr., of the Department of Pharmacology, School of Pharmacy, will be done concurrently on whole extracts, and, as they become available, on separate chemical fractions. In addition, correlative neurochemical analyses will be made in animal brain. We therefore hope to establish, on the basis of precise pharmacological characterization, which component(s) is active in producing the pharmacological effects shown by the whole extracts as well as the neurochemical correlates of these effects. With the achievement of these aims, a firm basis will have been established for the further studies necessary to achieve our long-range goals as stated in our overall working hypothesis.

B. Method of Procedure

a) Collection of Saliva Samples. Saliva specimens will be collected under controlled procedures previously established. Donors will comprise healthy adult, male, paid volunteers recruited from the university staff and/or the student body. Samples for analysis will be collected both as whole saliva and as duct saliva drawn differentially by means of duct cap devices as routinely used in this laboratory. All specimens will be collected approximately at the same time of day (mid-morning), over timed intervals, and transferred directly into chilled graduated receptacles containing measured amounts of ethanol so that the final sample will comprise a solution of saliva in 70% ethanol. This procedure serves both to curtail bacterial action and to provide precipitation of protein which, with appropriate addition of acetone and acidification to pH 4.5, is a necessary preparatory step for the subsequent ion-exchange column separation procedure. Collections will be made both with and without stimulation. The latter will be induced, as indicated by the specific experimental conditions, by passive chewing of paraffin for collection of whole saliva, controlled application of lemon juice to the tongue for duct saliva, or a single subcutaneous injection of 3-4 mg of urecholine (Bethanicol chloride, Merck) under appropriate supervision as in previous studies. Complete records will be made of subjects' age, height, weight, smoking habits, general state of health and nutrition as well as gross subjective feelings and affect prior to and during specimen collection. Candidates will be instructed to refrain from smoking for at least 2-3 hours prior to collection, and from the ingestion of

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alcohol and all drugs (especially aspirin or psychotropic tranquilizers, etc.) for at least 24 hours prior to specimen collection. Those requiring medication of any kind for any reason will not be accepted.

b) Preparation of SF extracts. The procedure to be used is essentially that of Kakimoto & Armstrong (2) as applied by Boulton (3) for the extraction of phenolic amines from urine, and modified by us for application to saliva. Saliva samples in 70% ethanol at pH 4.5 are centrifuged and filtered to remove precipitated protein, then applied to a column of appropriately pretreated Dowex 50W resin in the H^+ form. Conjugated compounds, neutral and most of the basic aliphatic substances are removed by appropriate washings with water and sodium acetate. The aromatic amines retained on the resin are eluted with N/1 NH_4OH in 65% ethanol and the eluate taken to dryness in vacuo at $40^\circ C$. The dried residue is extracted into absolute ethanol and centrifuged to remove any insoluble material. The clear supernate is quantitatively divided into 2 portions, one for quantitative spectrofluorometric examination at maximal activation & fluorescence and for thin-layer chromatographic (TLC) separation, and the other for reconstitution into aqueous solutions for testing in animals.

c) Chemical Separation and Identification of SF Extracts: Extracts prepared as described above will be concentrated to small volume, applied to silica gel thin-layer plates, and developed under standard conditions using the appropriate solvent systems as worked out in our previous studies, to ascertain the R_f properties of various extract components. Information as to chemical structure of individual spots will be sought by the use of various specific color developing agents for comparison with authentic phenylethyl- and phenylethanolamines and their derivatives used as reference standards. Eluted fractions of extracts striped on plates and located in this manner will be rechromatographed singly and in combination with reference compounds and further examined for absorption spectra, spectrofluorometric characteristics, as well as infra red spectroscopic patterns using procedures such as those described by Kirschenbaum and Parker (4).

d) Pharmacological Evaluation: The following pharmacological procedures will be conducted to ascertain the nature of SF whose initial screening characteristics suggest tyramine-like activity in mice:

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1. Hexobarbital sleeping time: Because preliminary screening studies have revealed that SF can potentiate hexobarbital sleeping time in mice and also that nicotine on the one hand and tyramine on the other can inhibit and prolong sleeping time respectively, the following experiments will be executed to elucidate the underlying mechanisms involved in sleeping time alteration and to determine whether SF elicits effects comparable to those produced by tyramine under similar conditions.

Treatment regimen:

	Measurement of:
low doses of nicotine + SF + hexobarbital)	duration of sleep
low saline control " ")	
high doses of nicotine + SF + hexobarbital)	" " "
high saline control " ")	
low dose(nicotine) + tyramine + hexobarbital)	" " "
low saline control " ")	
high dose(nicotine) + tyramine + hexobarbital)	" " "
high saline control)	

If tyramine and SF responses are similar in the presence of low or high doses of nicotine with respect to alterations in sleeping time, then confirmation of tyramine-like activity is made with respect to its relationship in the presence of a ganglionic blocking agent. If sleeping time is shortened in the presence of nicotine and SF as compared to saline and SF, the recognition of central neural sites for the action of SF will be indicated. However, nicotine stimulates then causes ganglionic depression; therefore, it is necessary to administer a pure (depressant only) gangliolytic agent in place of nicotine to determine that ganglionic depression instead of stimulation is responsible for the inhibition of the central effects induced by SF in the presence of hexobarbital. Thus:

	Measurement of:
Hexamethonium chloride + tyramine + hexobarbital)	duration s.t.
saline control " ")	
Hexamethonium chloride + SF + hexobarbital)	" "
saline control)	

If sleeping time is shortened significantly when either tyramine or SF is administered in the presence of C₆, this will indicate that ganglionic depression centrally is responsible for the inhibition of tyramine or SF potentiation of hexobarbital sleeping time rather than initial stimulation caused

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by nicotine in the earlier experiment.

2. Isolated Mercenaria mercenaria heart studies:

Previous studies utilizing the isolated heart of Mercenaria mercenaria suspended in sea water and attached to a recording device have revealed that successive administrations of norepinephrine rapidly cause the development of tachyphylaxis characterized by first a negative inotropic response that shortly disappears by the third or fourth administration (5). This procedure affords an excellent means by which the pharmacological similarities of tyramine and SF can be compared or contrasted.

PROCEDURES: The isolated heart is suspended in a 40 ml. chamber containing sea water at room temperature (24-30°) and aerated with air. Successive administrations of either tyramine at constant dosage levels or SF will be made at intervals governed by the time required for normalcy of contractions to return. In addition, the prior administration of tyramine followed by known tachyphylactic doses of norepinephrine (NE) and SF followed by NE will be studied to ascertain whether deviations in NE-induced tachyphylaxis occur.

3. Influence of SF and Tyramine on Sodium Nitro-Prusside and Hypothermia in Mature Mice:

We have recently demonstrated in our laboratory that oxotremorine and sodium nitroprusside each produce hypothermia in mature mice via a different mechanism (6). The parasympathomimetic, pilocarpine, did not alter oxotremorine-induced hypothermia, but partially inhibited that produced by sodium nitroprusside. Conversely, atropine inhibited oxotremorine-induced hypothermia, but failed to modify that elicited by sodium nitroprusside.

PROCEDURE: Mature mice will be divided into groups and treated as follows:

Rectal temperatures recorded immediately prior to start of treatment. 1-SF injection s.c., 15 minutes later rectal temperature recorded, oxotremorine admin. i.p., 15 minutes later, final rectal temp. recording; 2-tyramine injected, s.c., 15 minutes later rectal temperature recorded, oxotremorine admin., i.p., then

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15 minutes later, final rectal temp. recorded.
The procedure is repeated using sodium nitroprusside
in place of oxotremorine.

The influence of tyramine on either sodium nitroprusside- or oxotremorine-induced hypothermia has not been studied by us. Therefore, if comparable results are obtained with each hypothermic agent, following the injection of SF or tyramine, further proof of their close pharmacological characters will accrue.

4. Influence of SF and Tyramine on Nicotine Convulsions and Tremor in Young Mice.

The central action of nicotine in eliciting both convulsions and tremor is well known, and has found use in the evaluation of anti-convulsive as well as anti-tremor properties of many pharmaceutical preparations. This approach will be applied to testing SF and tyramine for relative anti-nicotine action. Methods of procedure using mice have been reported by a number of authors (7-11). These methods will be applied as follows:

PROCEDURE: Mice (18-26 gm) will be divided into groups and treated as follows:

1. Nicotine at low doses intracerebrally...observe tremor
SF + nicotine " " " " "
Saline controls..... " effects of i/c
injection
2. As in 1 above but using tyramine instead of SF..observe tremor
3. Nicotine at convulsive doses i/cobserve
convulsions
SF + nicotine " " " " "
" "
Saline control..... "
4. Ditto using tyramine instead of SF'.....observe
convulsions

e) Neurochemical Studies: The following procedures are intended to provide some neurochemical parameters of the pharmacological data from the previous section, and at the same time, to furnish some points of departure for future more detailed studies. Biochemical observations will be confined to the

measurement of norepinephrine (NE), dopamine (DPA) and serotonin (5HT) in extracts of whole brain homogenates of mice according to the method of Snyder, Axelrod and Zweig (18) as modified by Welsh & Welsh (12) and Leonard & Shallice (13). These estimations will be made in groups of mice injected according to the respective treatment schedules used for the pharmacological evaluation procedures. Because hexobarbital sleeping-time potentiation has been related to the brain amine-depletion effects of reserpine, uptake and release mechanisms become important neurochemical considerations with respect to our study of SF and its 'antagonist', nicotine. Accordingly, the above experiments will be extended to include observations on the effects, on the three brain amines mentioned, of SF, p-tyramine and derivatives, and nicotine. For this purpose, the experimental preparation will comprise mice pre-treated with reserpine, and in addition, with 4- α -dimethyl-m-tyramine (H77/77), a compound which has been found to displace brain catechol amines - particularly NE - and utilizes the reserpine-resistant uptake mechanisms to effect this displacement (19,20,21). This approach has been utilized recently by Leonard & Shallice (13) for studying the effects of phenylethylamines in brain and provides a suitable procedure for our comparative studies of SF, p-tyramine and nicotine.

C. Significance of this Research

This work concerns primarily a study of the relationship between autonomic nervous system activity and the biogenic amines in their role(s) as neurohumoral agents. Apart from the academic significance of our original discovery indicating the presence of such compounds in human saliva (15), the quantitative changes in their concentration after cholinergic stimulation immediately suggests a possible compensatory release of adrenergic amines in response to a cholinergic stimulus. In addition, such consideration would indicate a means of measuring, in humans, comparative autonomic responsivity to controlled cholinergic challenge by low doses of accepted pharmacological agents (see attached reprint of ref. 15 illustrating the marked differences between healthy controls and patients with severe alcoholism in their responses to urecholine challenge). In other words, these findings, substantiated as to interpretation by the proposed extended studies, could provide a relatively simple approach to the biochemical evaluation of an important aspect of autonomic status in terms of changes in the biogenic amines, which in themselves play an important part in the regulation of activity within the nervous system. The presence of SF in

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the submaxillary gland as well as in the saliva could be expected to reflect cellular activity in a gland which in turn, develops embryologically from the same oropharyngeal mucosa as does the anterior hypophysis. In this context, the salivary gland and its secretory products would appear to be an organ of choice for studying the neurochemical correlates of autonomic function in man. Further significance would stem from the application of these extended studies to the elucidation of the central actions of nicotine. In addition to the direct evidence stemming from our neurochemical studies of nicotine in animal brain, useful information on the autonomic effects of this drug - absorbed from the oral mucosa - could be obtained from tests in humans smoking tobacco under controlled conditions. The significance of the concept involving the oral absorption of neuroactive salivary components to fulfill homeostatic interactive functions at extra-oral sites, as stated in our overall working hypothesis, is that it presents a hitherto unrecognized role of the salivary glands as participants in regulatory neural mechanisms - an important new facet of oral biology. A fuller significance of these various aspects is expected to emerge from the extended research of this proposal.

D. Space and Facilities Available

The Biochemistry Department at the Temple University School of Dentistry has some 2,000 sq. ft. of newly renovated research space available in two large rooms with the use of a working cold room, fume hoods, and vented space for various types of chromatography, as well as offices and secretarial assistance.

The equipment available in the department includes: one Spinco RC-2B refrigerated ultra-centrifuge, two rotors (SW-25.1 and a 65), two Servall refrigerated centrifuges (one RC-2 and one SE-1), two size 2 ICI centrifuges kept in a cold room, and various table top centrifuges.

There are two Gilford 2000 recording spectrophotometers, one Perkin-Elmer Hitachi spectrophotometer, one Spectronic 505 spectrophotometer, one Spectronic 20, and two Coleman Junior visible spectrophotometers. There is also an Aminco-Rosett Recording Fluorimeter, and an Evans Atomic Absorption spectrophotometer.

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There are three fraction collectors, three expanded scale pH meters, various microscopes, one Frieden bench top computer and a Lago-marsino calculator, gel and strip electrophoresis equipment, thin-layer and paper chromatograph, three 18-station Warburg respirometers, one of which is refrigerated, two recording oxygen electrodes and a Radiometer carbon dioxide monitor, various micro-, semimicro- and macro balances, three mini-pumps and various columns for column chromatography, two lyophilizers, and a gradient former and eluter for the ultracentrifuge.

Available for our use within the Dental School are electron microscope service, histology service, a complete department for photography, art, and illustration, extensive animal care facilities, a complete radioisotope laboratory, a nuclear magnetic resonance service, and the advice and assistance of the staff of a large, well-functioning dental school. Pharmacological facilities are available within the School of Pharmacy.

The Aminco-Bowman Spectrophotofluorometer and Recorder are the only additional pieces of permanent equipment required for this work. The instrument presently available to us is on temporary loan. A new instrument is consequently being requested under the present budget.

2. Supporting Data

A. Previous Work by the Investigator Related to This Project

Our previous findings of immediate relevance to the present studies have been outlined in the previous Section 1-A. Details appear in reference No.'s 15-17 below, and in appended figures. Other relevant studies include experiments in rats - extending those of Schneyer & Schneyer (22,23) - which indicated significant increases in submaxillary gland amylase activity concomitant with immobilization stress without physical injury per se, but with resultant severe gastrointestinal ulceration. These unpublished experiments (25) illustrate further the chemical alterations in the salivary gland concomitant with augmented autonomic activity, whether induced by pharmacological agents (22) or by the psychological stress of immobilization.

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B. Pertinent Literature References

Our original finding of SF in human whole saliva has been confirmed by Hamby, et al. (24). Neuroactive substances in saliva and salivary glands of animals have been widely recognized for many years. Among those relevant to our studies are the early reports of Cattell, et al. (28) describing a neurohumoral agent with the properties of "adrenin" from cat submaxillary glands. The classical studies of Babkin and his associates (29) include extensive reports on various vasoactive substances (other than bradykinin or kallikrein) which vary in activity with autonomic stimulation. Similar findings have been reported by Hilton & Lewis (30). Stromblad (31,32) has described a monoamine oxidase acting upon tyramine substrate in the submaxillary glands of humans and animals. Most of the studies in human salivary gland function relative to the ANS have been restricted to salivary flow rate, and to some extent, electrolyte changes, although Giddon & Lisanti (33) have reported the occurrence of a cholinesterase-like substance in the parotid saliva of 'normal' and psychiatric patients. Of interest relative to biogenic amines is the report of Selye, et al. (34) demonstrating a five-fold increase in size of rats' submaxillary glands following chronic treatment with isoproterenol. This growth-stimulating action is reminiscent of the nerve-growth factor (NGF) effect upon sympathetic nerve tissue by extracts of mouse submaxillary glands reported by Levi-Montalcini & Cohen (35). Evidence relating to extra-oral sites of action of submaxillary gland products is seen in various accounts of the ostensible "hormone activity" of submaxillary gland tissue implants in the sella of the hypophysectomized dog by Alvarez-Buylla (37) in which the effects of hypophysectomy were almost entirely reversed in the presence of the implanted salivary gland. Evidence for a non-exocrine function of the submaxillary gland is presented in the growth experiments of Narasimhan & Ganla (36) in mice, rats, dogs and monkeys, and Godlowski & Calandra (39,40) have described an "insulin inhibitor" factor from dog submaxillary glands, thus suggesting an endocrine function for these gland structures. Our concept of the possible absorption of SF into the blood stream through the oral mucosa receives support from the recognized use of the sublingual route of administration of a variety of compounds including various steroid hormones in endocrinopathies, nitroglycerine in cardiac disorders, and isoproterenol in asthma. A direct pathway from the oral cavity to the brain has been described by Kare (38).

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REFERENCES

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C. Other Pertinent Publications (V.J. Schenker, Principal Investigator)

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(with L.S. Maynard)

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Nature, 196:575, 1962.
(with L.S. Maynard)

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A BIOCHEMICAL CORRELATE OF AUTONOMIC ACTIVITY: THE EFFECT OF BETHANECHOL ON SERUM AMYLASE IN DOGS.

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(with R.M. Engelman, P. Polimeni, M. Stucker, A. Riddick, A.C. Schenker and J.H. Stuckey)

ADRENAL HORMONES & AMINE METABOLISM IN ALCOHOLISM.

Psychosom. Med., XXVIII, No. 4: Part II 564-569, 1966.

(with A.C. Schenker, D. Kissin & L.S. Maynard)

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(with B. Kissin, L.S. Maynard & A.C. Schenker)

EFFECTS OF ETHANOL ON THE ENDOCRINES.

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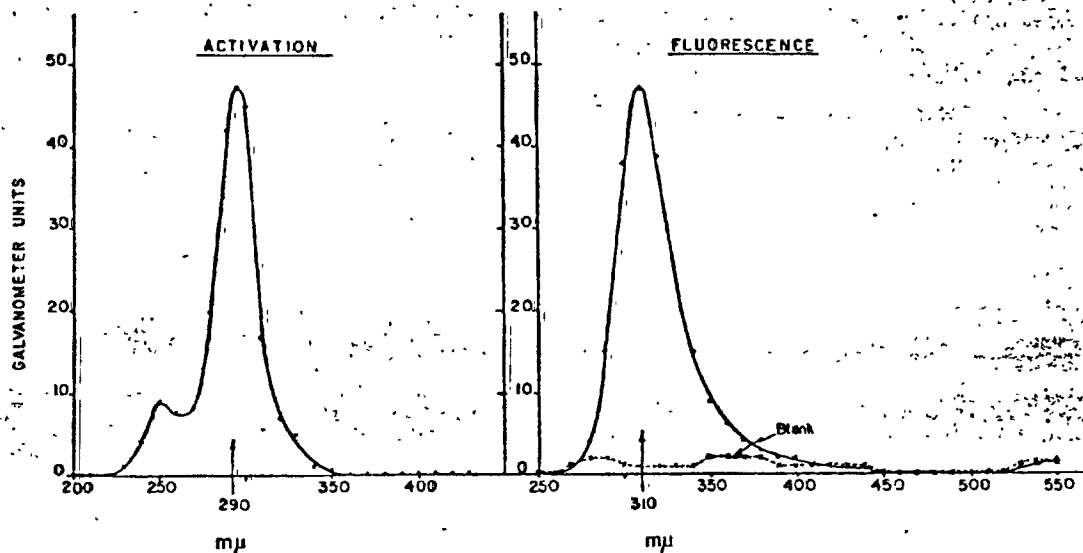


Figure 1. Activation and fluorescence spectra of the salivary factor (SF) extracted from human mixed saliva. Extracts of homogenized human submaxillary glands show identical spectra. Maximum fluorescence: 310 mμ, maximum activation 273 mμ (290 mμ corrected).

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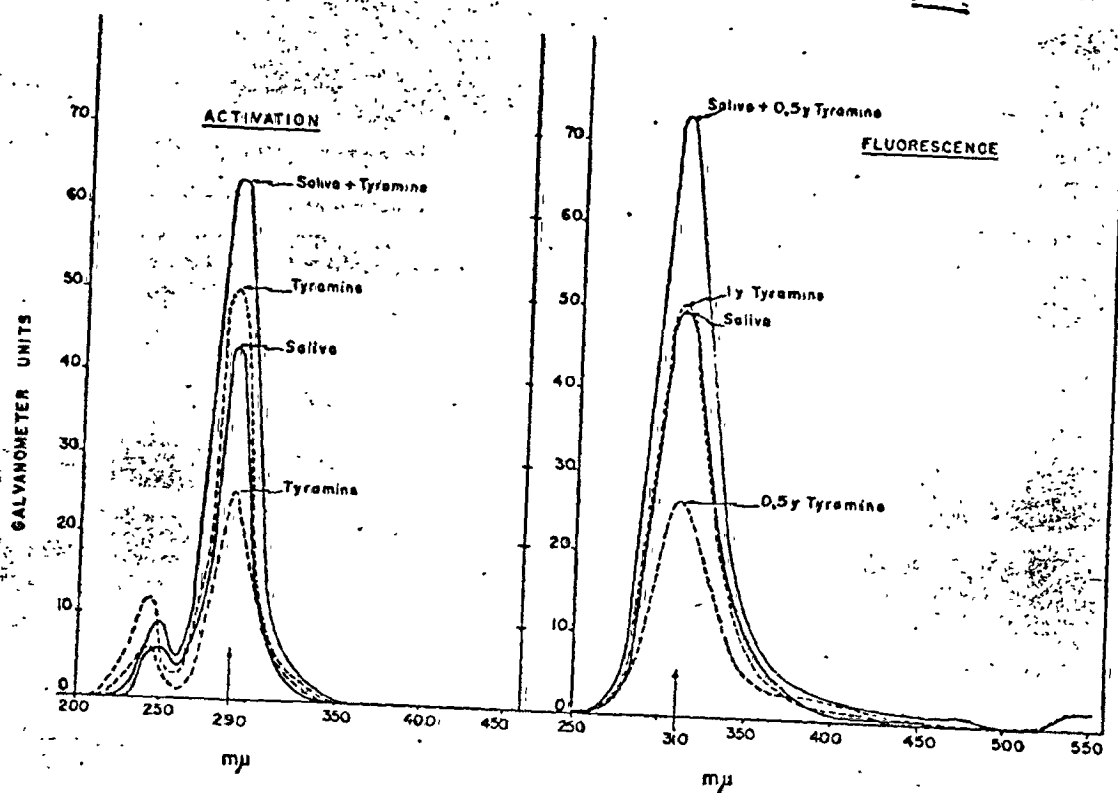


Figure 2. Comparative maximal activation and fluorescence curves of p-tyramine, and whole saliva with and without added p-tyramine all identically extracted. Note similarity between the salivary factor (SF) and authentic p-tyramine with activation at 290 mμ (corrected = 275 mμ) and fluorescence at 310 mμ.

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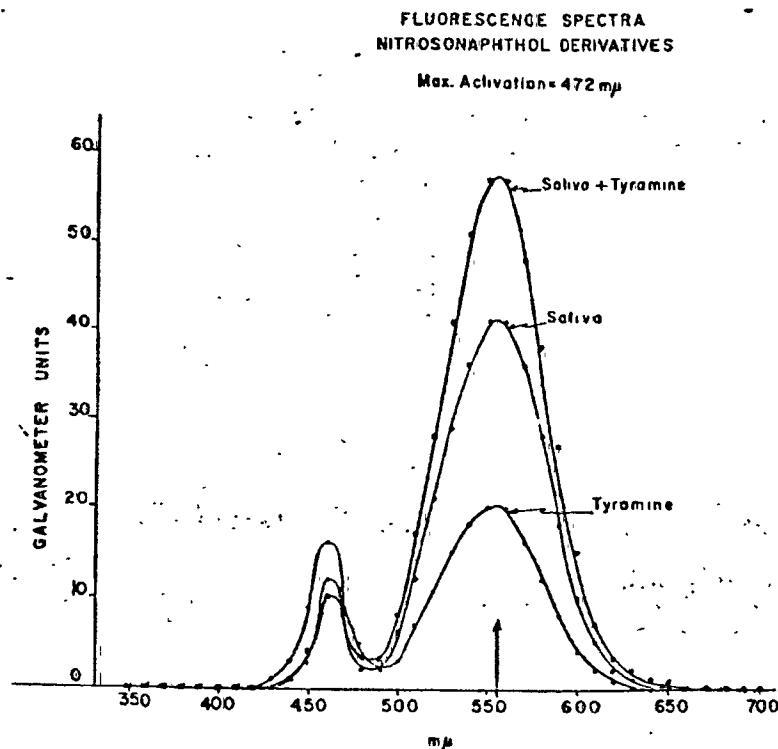


Figure 3. Fluorescence spectra of nitrosonaphthol- HNO_3 derivatives of p-tyramine and human whole saliva extracts, singly and in combination. Maximum fluorescence: 550-560 mμ, maximum activation 472 mμ. Note correspondence of curves for these derivatives.

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TABLE I

EFFECT OF SALIVA EXTRACT ON HEXOBARBITONE HYPNOSIS

Minutes	Sleeping	Time*
Blank Extract † plus Hexobarbitone		Saliva Extract ‡ plus Hexobarbitone
20.3 ± 8.5 (10)		46.8 ± 6.8 (10)

- * Interval between Loss & Recovery of Righting Reflex
† Water Blank carried through entire extraction procedure
‡ 0.4 ml Extract, i/v, 17 minutes before evipal (100mg/Kg) i/p.

Table 1. The effects pre-treatment with extracts containing SF upon the central action of hexobarbitone. Note the greater than 2-fold increase in sleeping time of treated animals over their controls.

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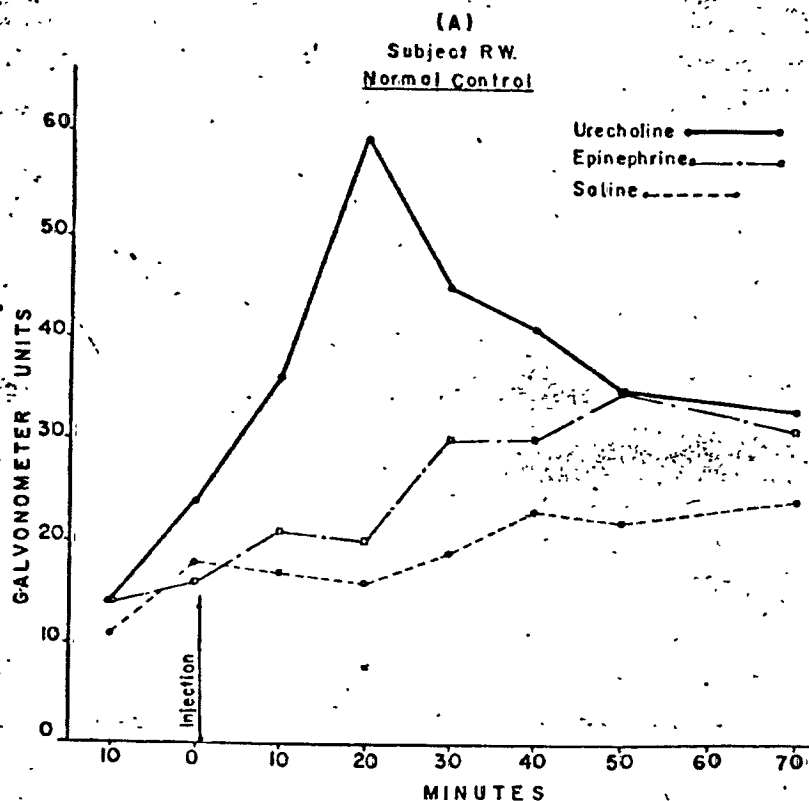


Figure 4. Representative response patterns of changes in SF secretion rate in a healthy male subject following the injection of 4 mg. urecholine (Bethanecol Chloride, Merek); 0.3 ml of 10^{-3} epinephrine (Adrenalin Chloride, Parke Davis); and physiological Saline as control. Note marked response curve after urecholine.

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SF in HUMAN WHOLE SALIVA

EFFECT OF URECHOLINE & ATROPINE ON SECRETION RATE

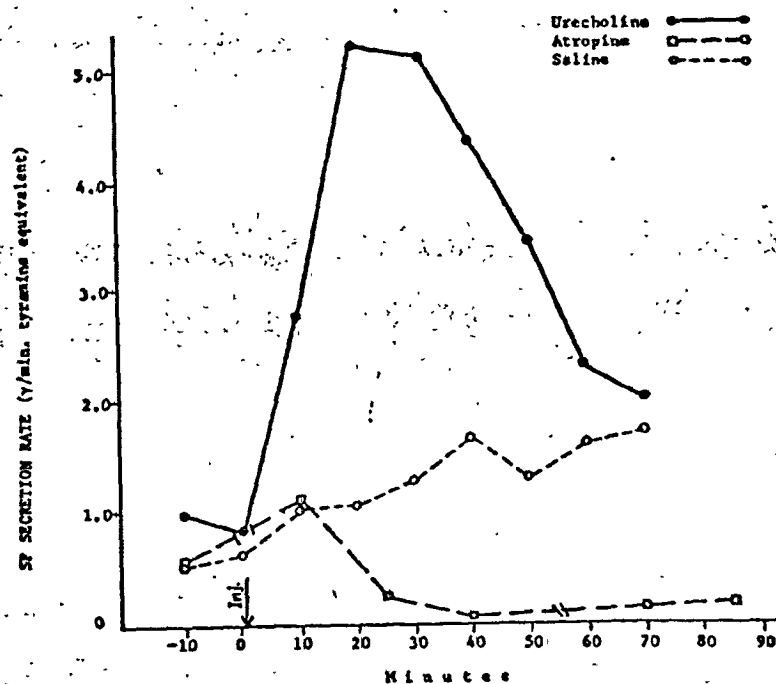


Figure 5. Changes in SF secretion rate in response to injections of urecholine 4 mg., atropine 0.64 mg, and saline control in a healthy adult male subject. Note the opposite effects of atropine and urecholine.

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EDUCATION:

St. Michael's Academy, Montreal, Canada
 Hosco Prep. School, Montreal, Canada
 Ecole Technique de Montreal - (Dipl. Tel. Comm'n.;
 First Award Final Year Thesis: Silver Medal for
 Second Highest Aggregate Standing.)
 McGill University - B.Sc. Degree
 McGill University - Ph.D. Degree (cum laude)
 in Experimental Medicine.

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POSITIONS HELD:

1927 - 1932: Out-Plant Engineering, Bell Tel. Co. of Canada.
 Estimating Engineer, Northern Elec. Co.,
 Montreal, Canada.

1933: Asst. Statistician (part-time) Dept. Social
 Research, McGill University.

1934 - 1935: Laboratory Technician, Dept. Exp'l Medicine,
 McGill University, Asst. to Dr. J.J. Day in
 the laboratories of Prof. B.P. Babkin -
 Gastroenterological research studies in dogs.

1937 - 1938: Research Assistant, Dept. Anatomy, McGill
 University - Research Studies on Adrenocortical
 Aspects of the "Alarm Reaction" under the
 direction of Dr. Hans Selye. Research Asst.,
 Biol. Labs.; Ayerst, McKenna and Harrison, Ltd.,
 Montreal, Canada, under direction of
 Dr. A. Stanley Cook.

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- 1939 - 1940: Research Asst., Dept. of Anatomy, McGill University - Studies of effects of the "Alarm Reaction" on Basal Metabolic Rate in Animals - under direction of Dr. Hans Selye.
- 1940 - 1941: Resumed former position as Research Asst. at Ayerst, McKenna & Harrison Laboratories.
- 1945 - 1948: Research Associate, Dept. Medicine, McGill University. Later, Research Fellow, McGill Univ. Clinic, Royal Victoria Hospital. Studies on Metabolic and Nutritional Aspects in Patients after Trauma and Drug Convalescence - Under direction of Prof. J.C. Meakins and Dr. J.S.L. Brown.
- 1947: Lecturer, Dept. of Medicine, McGill Univ. Research Fellow, University Clinic, Royal Victoria Hospital - Head, Nutrition Research Laboratory and Member of Attending Staff, Dept. Medicine, Royal Victoria Hospital.
- 1948 - 1951: Assistant Professor of Medicine, McGill Univ. (ibid)
- 1951 - 1953: Staff Member, Worcester Foundation for Experimental Biology, Shrewsbury, Mass. (Studies on adrenocortical function in schizophrenia and on biogenesis of adrenocortical hormones, with Drs. G. Pincus, O. Hocht, and H. Hoagland.
- 1953 - 1957: Assistant Professor Psychiatry (Biochemistry), State University of N.Y., College of Medicine, Brooklyn, N.Y.
- 1958 - 1965: Associate Professor of Psychiatry - Director, Biochemical Research Laboratory, State Univ. of N.Y., College of Med.
- 1965 - 1968: Associate Professor of Biochemistry, Albany College of Medicine, Union Univ. - Chief, Biochemistry, Psychiatry & Aging Research Laboratories, Veterans Administration Hospital, Albany, N.Y.
- 1968 - 1970: Research Biochemist, Affective Disease Research Unit, Veterans Administration Hospital, Philadelphia, Pa.
- 1972 - Research Professor of Biochemistry, Dept. of Biochemistry, Temple University School of Dentistry Philadelphia, Pa.

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RESUME OF AREAS OF RESEARCH

BIOCHEMICAL & ENDOCRINE FACTORS IN ALCOHOLISM

Adrenal Hormones & Amino Metabolism in Chronic Alcoholics.
Effects of Ethanol on Amine Metabolism in Alcoholism.
Effects of Ethanol on the Endocrines.
(Publ. #53, 54, 58)

Monoamine Oxidase, in vitro Inhibition (Publ. #46)
Decarboxylase Activity in vivo (misc) (" #45)
Pulmonary Respiration Pattern: Effects of Ethanol and
Chlorpromazine in Chronic Alcoholics. (Publ. #40, 44)
Acute Effects of Ethanol on Adrenocortical Function, Liver
Disease, & Other Physiological Functions in Alcoholic
Patients. (Publ. #36, 37, 38)

BIOCHEMICAL & PSYCHOLOGICAL STUDIES (Biochem. Psychopharmacol.)

Monoamine oxidase Inhibition & Antidepressive Correlates
in Psychiatric Patients. (Publ. #39)
Biochemical Correlates of Autonomic Function: Salivary
Factor; Response to autonomic activity in humans.
(Publ. #35, 51, 43)

ADRENOCORTICAL HORMONES

New Methods of Bioassay in rats. (Publ. #1, 30)
The nature & biogenesis of adrenocorticoids; adrenal perfusion
studies. (Publ. #26, 27, 28, 29, 31, 32, 33)
Studies in rats on the effects of the "Alarm Reaction" on
basal metabolic rates. (Publ. #2, 3)

METABOLIC ASPECTS OF CONVALESCENCE & WOUND HEALING

Protein Metabolism, Nitrogen Balance, Nutrition, & Adreno-
cortical Function in patients with disease & after acute
injuries. (Publ. #5 thru #24)
(Ph.D. Thesis)

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MEMBERSHIPS :

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P U B L I C A T I O N S

VICTOR J. SCHENKER

1. A NEW & RAPID METHOD FOR THE ASSAY OF THE HORMONE OF THE ADRENAL CORTEX.
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2. THE NON-PROTEIN NITROGEN CONTENT OF PLASMA DURING ADAPTATION TO VARIOUS STIMULI.
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3. FORMATION OF IRON-PIGMENT LYMPH NODES IN THE RAT.
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4. SOME EFFECTS OF DICOUMARIN - "HAEMORRHAGIC FACTOR" IN VIVO & IN VITRO STUDIES.
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5. NITROGEN METABOLISM & 17-KETOSTEROIDS IN PATIENTS AFTER BURNS & FRACTURES.
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6. EFFECTS OF ACTH ADMINISTRATION IN HEALTHY SUBJECTS: (I) GLUCOSE TOLERANCE, NITROGEN BALANCE, & URINARY 17-KETOSTEROIDS. (II) EFFECT OF ISO-CALORIC INCREASES IN DIETARY PROTEIN ON RESPONSE TO ACTH.
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10. SOME METABOLIC ASPECTS OF DAMAGE & CONVALESCENCE.
Jour. Clin. Invest., 23:932, 1944.
(with J.S.L. Browne & J.A.F. Stevenson)
11. CHANGES IN NITROGEN METABOLISM AFTER DAMAGE.
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OBSERVATIONS IN 55 SURGICAL CASES RECEIVING PROTEIN HYDROLYSATES.
Assoc. Comm. on Army Med. Res., Proc., 8th Meeting, No.C6348,
1945.
(with J. Clark)
13. NUTRITION IN CONVALESCENCE.
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(with J.A.F. Stevenson & J.S.L. Browne)
14. THE CHARACTERISTIC PATTERN IN THE METABOLISM OF NITROGEN AFTER
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15. NITROGEN METABOLISM IN CHRONIC DISEASE.
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16. RIBOFLAVIN METABOLISM AFTER TRAUMA & DURING CONVALESCENCE IN MAN.
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(with W.A. Andreae)
17. STUDIES IN THE HOURLY RATE OF NITROGEN EXCRETION IN PATIENTS AFTER
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escence, Trans. 12:31, 1946.
18. NITROGEN-SPARING ACTION OF INSULIN IN ADDITION TO HIGH NON-PROTEIN
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20. METABOLIC & NUTRITIONAL STUDIES ON DEBILITY IN PATIENTS WITH
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XVII Internat. Physiol. Cong., Oxford, England, 1947 (abstr. p. 229) (with J.S.L. Browne & L.G. Johnson)
22. HIGH PROTEIN DIETS.
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(with O. Hechter, R.P. Jacobsen, R. Jeanloz, H. Levy, C.W. Marshall, & G. Pincus)
29. THE NATURE & BIOGENESIS OF THE ADRENAL SECRETORY PRODUCT.
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1. Mann, David E., Jr., "Effect of orally administered potassium iodate on blood sugar response to thiourea," *Proceedings of the Society for Experimental Biology and Medicine*, **73**, 657-658 (1950);
2. Zarrow, M.X., Denison, M.E., Rosenberg, B., and Mann, D.E., Jr., Neher, G.M., "Effect of insulin and epinephrine on the eosinophil and blood glucose levels in sheep; lack of diurnal rhythm," *The American Journal of Physiology*, **171**, 636-640 (1952);
3. McCreesh, A. H., and Mann, David E., Jr., "The effect of orally administered sodium iodide and sodium iodate on blood sugar response to thiourea in the rat," *Journal of American Pharmaceutical Association, scientific edition*, **47**, 56-57 (1958);
4. Fujita, T., and Mann, David E., Jr., "Further studies on 1-arterenol tachyphylaxis in the isolated heart of *Venus mercenaria*," *Journal of the American Pharmaceutical Association, scientific edition*, **47**, 90-93 (1958);
5. Gautieri, R.F., and Mann, David E., Jr., "Determination of the minimal carcinogenic dose₅₀ of methylcholanthrene on mouse epidermis," *Journal of the American Pharmaceutical Association, scientific edition*, **47**, 350-353 (1958);
6. Goldenberg, M.M., and Mann, David E., Jr., "The antidotal effectiveness of sodium cobaltinitrite in antagonizing cyanide poisoning in albino mice," *Journal of the American Pharmaceutical Association, scientific edition*, **49**, 210-212 (1960);
7. Gautieri, R.F., and Mann, David E., Jr., "Effect of gonadectomy and estradiol benzoate administration on the minimal carcinogenic dose₅₀ of methylcholanthrene on mouse epidermis," *Journal of Pharmaceutical Sciences*, **50**, 556-560 (1961);
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10. Orzechowski, R.F., and Mann, David E., Jr., "Effects of dl-, d-, and l-amphetamine on levarterenol tachyphylaxis in the isolated heart of *Venus mercenaria*," *Journal of Pharmaceutical Sciences*, 52, 337-340 (1963);
11. Mancini, R.T., Gautieri, R.F., and Mann, David E., Jr., "Effect of cortisone on the minimal carcinogenic dose₅₀ of methylcholanthrene in albino mice," *Journal of Pharmaceutical Sciences*, 53, 385-388 (1964);
12. Orzechowski, R.F., Gautieri, R.F., and Mann, David E., Jr., "Effect of sodium nitrite and p-aminopropiophenone on the minimal carcinogenic dose₅₀ of methylcholanthrene on mouse epidermis," *Journal of Pharmaceutical Sciences*, 54, 64-66 (1965);
13. Orzechowski, R.F., Gautieri, R.F., and Mann, David E., Jr., "Effect of sodium cobaltinitrite on the minimal carcinogenic dose₅₀ of methylcholanthrene in albino mice," *Journal of Pharmaceutical Sciences*, 53, 388-391 (1964);
14. Harpel, Howard S., Jr., and Mann, David E., Jr., "Antagonism of dextro-propoxyphene poisoning in albino mice with nalorphine HCl, levallorphan tartrate, and methylene blue," *Journal of Pharmaceutical Sciences*, 54, 97-100 (1965);
15. Bagdon, W.J., and Mann, David E., Jr., "Promazine hyperthermia in young albino mice," *Journal of Pharmaceutical Sciences*, 54, 153-154 (1965);
16. Bagdon, W.J., and Mann, David E., Jr., "Factors modifying chlorpromazine hyperthermia in young albino mice," *Journal of Pharmaceutical Sciences*, 54, 240-246 (1965);
17. Thompson, R.S., Gautieri, R.F., and Mann, David E., Jr., "Effect of chronic oral administration of sodium cobaltinitrite and sodium nitrite on the minimal carcinogenic dose₅₀ of methylcholanthrene in albino mice," *Journal of Pharmaceutical Sciences*, 54, 595-598 (1965);
18. Kasirsky, G., Gautieri, R.F., and Mann, David E., Jr., "Effect of cobaltous chloride on the minimal carcinogenic dose₅₀ of methylcholanthrene in albino mice," *Journal of Pharmaceutical Sciences*, 54, 491-493 (1965);
19. Mann, David E., Jr., "Antagonism of propoxyphene poisoning in albino mice with nalorphine HCl, methylene blue, and tolonium chloride," *Journal of Pharmaceutical Sciences*, 56, 130-131 (1967);

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31. CHEMICAL TRANSFORMATIONS OF STEROIDS BY ADRENAL PERFUSION, I: PERFUSION METHODS.
Endocrinology, 52:679, 1953.
(with H. Levy, R. Jeanloz, C.W. Marshall, R.P. Jacobsen, O. Hechter, & G. Pincus)
32. CHEMICAL TRANSFORMATIONS OF STEROIDS BY ADRENAL PERFUSION, II: 11-DESOXYCORTICOSTERONE & 17-HYDROXY, 11-DESOXYCORTICOSTERONE.
Jour. Biol. Chem., 203:4333, 1953.
(with H. Levy, R. Jeanloz, C.W. Marshall, R.P. Jacobsen, O. Hechter, & G. Pincus)
33. CHEMICAL TRANSFORMATIONS OF STEROIDS BY ADRENAL PERFUSION.
Jour. Biol. Chem., 211, 2:867, 1954.
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34. SERIAL LIVER FUNCTION AND BLOOD STUDIES IN PATIENTS RECEIVING CHLORPROMAZINE.
New Eng. Jour. Med. 256:1, 1957.
(with R. Dickes & L. Deutsch)
35. STUDIES ON HUMAN SALIVA: A TYRAMINE-LIKE COMPONENT AND ITS RESPONSE TO AUTONOMIC STIMULATION.
Jour. Nerv. & Ment. Dis., 128(6):520, 1959.
(with Anne C. Schenker)
36. THE ACUTE EFFECTS OF ETHYL ALCOHOL & CHLORPROMAZINE ON CERTAIN PHYSIOLOGICAL FUNCTIONS IN ALCOHOLICS.
Quart. Jour. Studies Alcohol, 20:480, 1959.
(with B. Kissin & A.C. Schenker)
37. ADRENAL CORTICAL FUNCTION AND LIVER DISEASE IN CHRONIC ALCOHOLICS.
Am. Jour. Med. Sci., 238:344, 1959.
(with B. Kissin & A.C. Schenker)
38. THE ACUTE EFFECT OF ETHANOL INGESTION ON PLASMA & URINARY 17-HYDROXYCORTICOIDS IN ALCOHOLIC SUBJECTS.
Am. Jour. Med. Sci., 239:690, 1960.
(with B. Kissin & A.C. Schenker)
39. MONOAMINE OXIDASE INHIBITION AND ANTIDEPRESSIVE CORRELATES.
Proc. Third World Congress Psychiatry, Vol. I, pp. 642-649, 1961.
(with G. Marjerrison, P. Schlachet, N. Freedman, L.D. Hankoff, and D.M. Engelhardt)
40. ABERRATIONS IN THE PULMONARY RESPIRATORY PATTERN IN ALCOHOLICS & THE ACUTE EFFECTS OF ETHYL ALCOHOL & CHLORPROMAZINE UPON SUCH PATTERNS.

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Ibid., Vol. I, pp. 389-396, 1961.
(with A.C. Schenker & B. Kissin)

41. CEREBROSPINAL FLUID CATION LEVELS IN DELIRIUM TREMENS WITH SPECIAL REFERENCE TO MAGNESIUM.

Jour. Nerv. Ment. Dis., 134:410, 1962.
(with L.S. Glickman, S. Grolnick, A. Green, & A.C. Schenker)

42. EFFECTS OF ETHANOL ON MONOAMINE OXIDASE, PRELIM. STUDIES IN HUMANS & MOUSE TISSUES.

Proc. of Symposium on Biochem. Factors in Alcoholism, N.Y.
Acad. Med. Dec. 1961.
(with L.S. Maynard & I. Rothrock)

43. BIOCHEMICAL STUDIES IN HUMAN SALIVA: FURTHER OBSERVATIONS ON THE TYRAMINE-LIKE COMPONENT AND ITS RESPONSE TO AUTONOMIC DRUGS.

Biochem. Pharmacol., 12 (Suppl.):133, 1963
(with L.S. Maynard)

44. ACTION OF ATARACTIC DRUGS ON ANXIETY AS MEASURED BY RESPIRATORY PATTERN & PSYCHOLOGICAL ASSESSMENT.

In: Drugs & Respiration, Proc. Second Internat'l Pharmacol Meeting, Prague. Aviado & Palccok (Eds.), Vol. 11, pp. 129-
(with A.C. Schenker & E. Edelstein)

45. DECARBOXYLASE ACTIVITY IN VIVO: EFFECTS OF MORPHINE & ETHANOL PRETREATMENT IN MICE.

Int. J. Neuropharmacol. 2:303, 1964.
(with L.S. Maynard)

46. MONOAMINE OXIDASE INHIBITION BY ETHANOL IN VITRO.

Nature, 196:575, 1962.
(with L.S. Maynard)

47. A BIOCHEMICAL CORRELATE OF AUTONOMIC ACTIVITY: THE EFFECT OF BETHANECHOL ON SERUM AMYLASE IN DOGS.

Surgery, 56:394, 1964.
(with R.M. Engelman, P. Polimeni, M. Stuckey, A. Riddick, A.C. Schenker & J.H. Stuckey)

48. HYPERDIURESIS AFTER ETHANOL IN CHRONIC ALCOHOLICS.

Amer. Jour. Med. Sci. 1964, 248:660-669.
(with B. Kissin & A.C. Schenker)

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49. THE EFFECTS OF ETHANOL ON AMINE METABOLISM IN ALCOHOLISM.
Proc. 27th Internat'l. Cong. Alcohol & Alcoholism,
Frankfort/Main, Germany 1964.
(with B. Kissin & A.C. Schenker)
50. GLUTAMIC ACID METABOLISM IN VIVO: THE EFFECTS OF PRETREATMENT
WITH MORPHINE SULPHATE.
Biochem. Pharmacol., 13:1507, 1964.
(with L.S. Maynard)
51. AN APPARENT "PRIMATE SPECIFIC" SALIVARY AMINE.
Proc. Internat'l. Neurochem. Conference, Oxford, Eng. 1965.
(with L.S. Maynard)
52. EFFECTS OF STRESS ON TISSUE UPTAKE OF BIOGENIC AMINES: THE
MODIFYING INFLUENCE OF MORPHINE SULFATE & OF ALCOHOL.
Ibid., idem.
53. ADRENAL HORMONES & AMINE METABOLISM IN ALCOHOLISM.
Psychosom. Med., XXVIII, No. 4: Part II 564-569, 1966.
(with A.C. Schenker, D. Kissin & L.S. Maynard)
54. THE EFFECTS OF ETHANOL ON AMINE METABOLISM IN ALCOHOLISM.
In: Biochemical Factors in Alcoholism, pp. 39-52, R.P.
Maickel (Ed) Pergamon Press, Oxford & New York, 1966.
(with B. Kissin, L.S. Maynard & A.C. Schenker)
55. INDUCED HEMORRHAGIC HYPOTENSION: ITS EFFECT ON PLASMA 5-
HYDROXYTRYPTAMINE AND PLASMA 5-HYDROXYINDOLE ACETIC ACID
LEVELS.
Arch. Surg., 98:194-198, 1969.
(with D.J. Ducore, L. Schnipper & J.H. Stuckey)
56. PLASMA SEROTONIN LEVELS IN STORED HUMAN BLOOD.
Angiology, 18:535, 1967.
(with H.W. Strauss, R.B. Smith, P. Polimeni, A.C. Schenker
and J.H. Stuckey)
57. URINARY EXCRETION OF EPINEPHRINE, NOREPINEPHRINE, DOPAMINE AND
TRYPTAMINE DURING SLEEP AND WAKEFULNESS.
In: Psychopharmacologia (Berl.) 14:359-370 (1969)
(with Fredrick Baekland, Anne C. Schenker & Richard Lasky)
58. EFFECTS OF ETHANOL ON THE ENDOCRINES.
In: Internat'l Encyclopedia of Pharmacology and Therapeutics,
Section 20, Vol. 1, Chap. 10, J. Tromolieres (Ed) Pergamon
Press, 1970.

1003538920

FACULTY VITAE - DAVID E. MANN, JR.

BORN: Johnson City Tennessee, R

Education: Needham High School. R

Harvard College, R B.S.

Tufts College and Medical School, R

U.S.N.R., R

Boston University, R

Purdue University, M.S. (physiology); R

Purdue University, Ph.D. (physiology);

Employment: Laboratory technician, Boston Consolidated Gas Co., 1947

Assistant professor of physiology and pharmacology, Temple University
School of Pharmacy, 1950

Instructor in physiology, Purdue University (summer school), 1951

Associate professor of pharmacology; dept. chairman, Schools of
pharmacy and dentistry of Temple University, 1954

Professor of pharmacology, 1960

Society Membership:

REDACTED

Honors and Awards:

Lindback Award for Teaching, June, 1966

Certificate of Merit, Dictionary of International
Biography, July, 1967

Inclusion in 1970 edition of "Outstanding Educators of America"

President of the Temple Chapter of Sigma Xi, 1960-61

Biographical references:

Who's Who in the East

The Blue Book

Dictionary of International Biography

Two Thousand Men of Achievement

American Men and Women of Science

1003538921

Faculty Vitae - David E. Mann, Jr. (Continued)

GRANTS AWARDED:

<u>Title</u>	<u>Grantor Institution</u>	<u>Date Awarded</u>	<u>Amount</u>
1. "Effect of tobacco smoke and residues on methylcholanthrene-induced skin carcinogenesis"	Tobacco Industry Research Committee	9/24/54	5,500.00
Renewal		9/20/55	1,988.00
2. "Factors in development of 1-arterenol tachyphylaxis"	N I H	10/5/59	3,335.00
Renewal		10/1/60	3,335.00
3. "Methemoglobinemia and carcinogenesis"	N I H	1/1/62	15,850.00
Renewal		1/7/63	10,898.00
4. "Factors modifying chlorpromazine hyperthermia"	N I H	6/27/63	3,383.00
5. "Effect of vasodilators on perfused human placenta"	N I H	5/1/62	8,395.00
Renewal		5/1/63	8,346.00
Renewal		5/1/64	4,345.00
6. "Chronic methemoglobinemia and carcinogenesis"	Damon Runyon	6/1/63	5,500.00
7. "Modification of Teratogenicity by cobalt"	N I H	5/1/66	12,475.00
Renewal		5/1/67	12,475.00

INVENTIONS:

Two patents (one design patent)

Invented ejector seat used in James Bond film, "Goldfinger;" Popular Science, February, 1947 (page 110) and Popular Science, March, 1966 (page 13).

PublicationsLaboratory Manual:

"A Laboratory Manual of Pharmacology," by David E. Mann, Jr. First printed in 1954 and since then used at Temple University in the school of pharmacy and also at the pharmacy schools of the following universities: Montana, Texas, and excerpts at P.C.P.&S.

1003538922

PUBLICATIONS (Continued)

in lay journals:

1. "Death takes a holiday," Purdue Scientists, 1, 9-11 (1947);
2. "How to hunt whitetail deer," Hunting and Fishing, Dec., 58-60 (1947);
3. "The man behind the Nobel prize," Purdue Scientist, 1, 14-16 (1948);
4. "Recent developments in the field of antidiabetic drugs," The Pennsylvania Pharmacist, 32, 16-33 (1951);

Abstracts of scientific papers:

1. Mann, David E., Jr., and Hiestand, W.A., "The relative antidotal effects of certain organic compounds and potassium cyanide in the albino mouse," ANATOMICAL RECORD, 101, 744 (1948);
2. Mann, David E., Jr., Zupko, A.G., Hammond, P.V., and Rockhold, W.T., "The effects of Veratrum viride on the blood sugar level of the albino rat," ANATOMICAL RECORD, 105, 617 (1949);
3. Mann, David E., Jr., Zupko, A.G., Hammond, P.V., and Rockhold, W.T., "The effects of Veratrum viride on the blood sugar level of the albino rat," Proceedings of the Indiana Academy of Science, 59, (1950);
4. Mann, David E., Jr., and Zarrow, M.X., "Normal blood sugars in sheep and lambs," Federation Proceedings, 9, 84 (1950);
5. Mann, David E., Jr., and Hiestand, W.A., "Alloxan response following prolonged oral administration of thiourea in the rat," ANATOMICAL RECORD, 111, 578 (1951);
6. Mann, David E., Jr., "Effect of potassium iodide and potassium iodate on blood sugar response to thiourea," Journal of Pharmacology and Experimental Therapeutics, 110, 34 (1954);
7. Swanson, Jr., E. A., Ploumis, E., and Mann, David E., Jr., "Radiologic and histologic changes in the dental pulp chamber incident to experimental arteriosclerosis," American Journal of Anatomy (accepted for entry), May, 1973.

1003538923

Journal Articles (Continued) :

20. Kasirsky, G., Gautieri, R.F., and Mann, David E., Jr., "Inhibition of cortisone-induced cleft palate in mice by cobaltous chloride, " Journal of Pharmaceutical Sciences , 56 , 1330-1332 (1967);
21. Mann, David E., Jr., Gautieri, R.F., and Kasirsky, G., "Ionic hormonal precursor hypothesis," The Lancet , March 30th , 1968, p. 699;
22. Arcuri, P. A., and Mann, David E., Jr., "Effect of sodium fluoride and/or sodium iodate on blood sugar response to thiopental in the fasted rabbit," Journal of Pharmaceutical Sciences , 58 , 260-261 (1969);
23. Kasirsky, G., Sherman, W.T., Gautieri, R.F., and Mann, David E., Jr., "Cobalt-cortisone interrelationships in the induction and inhibition of cleft palate in mice, " Journal of Pharmaceutical Sciences , 58 , 766-767 (1969);
24. Burke, D.H., and Mann, David E., Jr., "Influence of several autonomic drugs on sodium nitroprusside and oxotremorine-induced hypothermia in immature and mature mice," Journal of Pharmaceutical Sciences , 59 , 1814-1818 (1970);
25. O'Hara , G.P., Mann, David E., Jr., and Gautieri, R.F., "Effect of cobalt chloride and sodium cobaltinitrite on the growth of established epithelial tumors induced by methylcholanthrene," Journal of Pharmaceutical Sciences , 60 , 473-474 (1971);

Temple Dental Alumni Review magazine:

1. "Dental Therapeutics, 2001," by David E. Mann, Jr., (in press).

AUDIO-VISUAL TAPES:

1. "The Ionic Hormonal Precursor Hypothesis," prepared as a 12-minute tape for TV viewing, embodies the experimental concepts backing our original hypothesis that present-day hormones originated from ions in primeval seas. This tape was one of 13 presented before the Sixth International Congress on Pharmacology, July, 1972, in San Francisco.
2. "Prescription Writing," a 9-minute tape prepared for teaching purposes in the dental school. It illustrates the fundamental principles involved in Rx writing.
3. "Use and Abuse of Local Anesthetics," a 30-minute tape which introduces the student of dentistry to the hazards encountered when local anesthetics are improperly used.

1003538924

Curriculum Vitae

Name: Robert L. Pollack

Address

Home Telephone Number:

REDACTED

Born:

Philadelphia, Pa.

Marital Status:

REDACTED

Military Experience: Hospital Corps, United States Navy, 1944-45

Education:

B.Sc. in Chemistry, R Philadelphia College of Pharmacy
and Science

B.Sc. in Bacteriology, R Phila. College of Pharmacy and
Science

M.Sc. in Bacteriology, R Phila. College of Pharmacy and
Science

Ph.D. in Biochemistry, R University of Tennessee Medical
Units Division, Memphis, Tenn.

Professional Experience:

Professor of Biochemistry and Chairman, Department of
Biochemistry and Nutrition, Temple University School
of Dentistry, 1962-present.

Consultant in Dental Biochemistry to the Veterans Administra-
tion Hospital, Philadelphia, 1962-1967

Senior Research Scientist, Eastern Regional Research
Laboratories, U.S. Dept. of Agriculture, Wyndmoor, Pa.,
1954-1962.

Adjunct Instructor in Chemistry, Drexel Institute of
Technology Evening Division, 1957-1962

Memberships:

REDACTED

Listings in Professional Directories:

American Men in Science

Leaders in American Science

Dictionary of International Biography

Who's Who in American Education

1003538925

Abstracts Presented at Meetings

1. An Amino acid-steroid conjugate excreted in urine. C.H. Eades, Jr., R.L. Pollack, and J.S. King, Jr., Federation Proc. 13, 201-202 (1954).
2. Respiratory activity of normal and bruised red tart cherry (*Prunus cerasus*). R.L. Pollack and C.H. Hills, Federation Proc. 15, 328 (1956).
3. Stabilizing apple cider by mild heat treatment. J.F. Robinson, R.L. Pollack, and C.H. Hills. 18th annula meeting, Institute of Food Technologists. Chicago, Ill. 1958.
4. The respiratory activity of the red tart cherry during growth. Robert L. Pollack and Claude H. Hills. IXth International Botanical Congress, Montreal, Canada, August, 1959.
5. A quantitative study of the nitrogenous components of the red tart cherry. R.M. Zacharius and R.L. Pollack. Oregon State University. Corvallis, Oregon, 1962.
6. The respiratory activity of lathrogen-treated strain "L" fibroblasts in culture. J.J. Aleo, R.L. Pollack, and G.R. Schacterle. 45th General Meeting, International Assoc. for Dental Research, March, 1967.
7. Substrate and cofactor effects on respiration of bovine dental pulp. R. L. Pollack, D. Green, and T. Rosett, 49th general meeting of the International Association for Dental Research, March, 1971.
8. Model systems for the study of oral tissue; gingival and lingual epithelium and dental pulp. T. Rosett, L. P. Gangarosa, R. L. Pollack, D. Green, and P. Garner. 55th annual meeting of the Federation of American Societies for Experimental Biology, April, 1971.
9. An individualized instruction procedure for a biochemistry laboratory experiment. T. Rosett and R. L. Pollack. Section on Learning Resources, American Association of Dental Schools, March, 1972.

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Publications

1. Determination of Free and Combined Amino Acids in Urine, Robert L. Pollack and C.H. Eades, Jr., Anal. Chem. 24, 2017 (1952).
2. Glucuronic Acid Conjugates of Aspartic and Glutamic Acids in Urine, Robert L. Pollack, and C.H. Eades, Jr., Science 119, 510-511 (1954).
3. Urinary Excretion of Fourteen Amino Acids by Normal and Cancer Subjects, C.H. Eades, Jr., and Robert L. Pollack, J. National Cancer Institute 15, 421-427 (1954).
4. Thermal Burns in Man. IX. Urinary Amino Acid Patterns, Charles H. Eades, Jr., Robert L. Pollack, and James D. Hardy, J. Clinical Investigation, 34, 1756-1759 (1955).
5. Studies on Cherry Scald. I. Relationship Between Bruising and Respiration in Water, Robert L. Pollack, C. Ricciuti, C.F. Woodward, and C.H. Hills, Food Tech. 12, 102-105 (1958).
6. Studies on Cherry Scald. II. Relationship Between Bruising and Respiration in Air. Robert L. Pollack, Claude H. Hills and R.T. Wittenberger, Food Tech. 12, 106-108 (1958).
7. A Rapid Method for Serum Uric Acid without Cyanide, G.F. Grossman, A. Grossman, E. Kravitz and R. L. Rollack, American Journal of Pharmacy 133, 213-218, 1961.
8. Respiratory Activity of the Red Tart Cherry (Prunus Cerasus) During Growth. Robert L. Pollack, Nancy Hoban, and Claude H. Hills, Proc. Am. Soc. Hort. Sci., 78, 86-95, 1961.
9. Self Mountable Support - U.S. Patent Application #270084. Filed April 2, 1963. Issued, August 24, 1965.
10. Modifications to the Autoanalyzer for the rapid recording of optical densities. A. Grossman, G.F. Groossmann, R.L. Pollack, and E. Kravitz, Anal. Biochem. 8, 124-126, 1964.
11. Respiration in bruised fruit tissue. Robert L. Pollack, Grafton C. Chase, and Joseph L. Rabinowitz, Atompraxis 11, Sept/Oct., 562-564, 1965.

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Robert L. Pollack
Curriculum vitae

12. Effect of Bruising and Aging on the Alcohol-Insoluble Solids of Red Tart Cherries. R.H. Golder, S.M. Levin, G. R. Schacterle, and R.L. Pollack. J. Agric. & Food Chem. 20, 680, 1972.
13. The Comparative Analysis of Diabetic and Non-Diabetic Saliva. Protein separation by disc gel electrophoresis. A.J. Finestone, G.R. Schacterle, and R. L. Pollack. Accepted for publication by the Journal of Periodontology.
14. Respiration of homogenates and crude mitochondrial fractions of bovine attached gingiva. T. Rosett, L.P. Gangarosa, E.L. Ashbridge, A. Belsky, G. Derenzo, H. Elder, R.L. Pollack, U. Sacco, and N. Tan. Archs. Oral Biol. 17, 1543-1550, 1972.
15. Respiration of homogenates and mitochondrial fractions of bovine dental pulp. T. Rosett, E. Ashbridge, A. Belsky, G. Derenzo, P.S. Garner, D. Green, R.L. Pollack, and N. Tan. Archs. Oral Biol. 17, 1691-1698, 1972.
16. A Simplified Method for the Quantitative Assay of Small Amounts of Protein in Biologic Material. G.R. Schacterle and R. L. Pollack, Accepted for publication in Analytical Biochemistry.

1003538928

#894 VALZELLI

1003538929

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

2.
Denied
December 18, 1972

Grant application No. 894

To: The committee comprising Drs. Bing, Cattell, and Jacobson

Subject: Luigi Valzelli, Professor, M.D., Istituto di Ricerche
Farmacologiche Mario Negri, Milan, Italy
New application No. 894
"Nicotine Effects Upon Aggressive Behavior"

History

Although the Council is well acquainted with this institution and investigator, no specific antecedent for this particular application is known. Application No. 894 requests \$21,748 for 1973-74. (Overhead requested less than entitlement, doubtless because of a clerical error).

One additional year is projected.

Documents Submitted

1. Application dated December 5, 1972 with enclosures A through D.
2. Enclosure E, No. 1 through No. 7, are publications going back to 1969. These papers appear in the list in enclosure C. Copies will be forwarded if you wish.

Comment

This applicant has collaborated with CTR grantee Walter B. Essman. Indeed this application was forwarded by Essman, ostensibly because of postal difficulties in Italy.

F.W.N.

F.W.N.

FWN:wg
Encls.

1003538930

THE JACKSON LABORATORY
BAR HARBOR, MAINE

MEMORANDUM

16 January 1973

To: DR. F. NORDSIEK
From: H. MEIER *HM*
Subj: APPLICATION NO. 894, "NICOTINE EFFECTS UPON AGGRESSIVE
BEHAVIOR", BY VOLZELLI AND MUSSINI

As I could not judge this application myself, I indeed called upon one of my colleagues who, as the reviewer stated, is in this "kind of business". Here is his verbatim and confidential report to me:

"As I mentioned to you over the telephone, I think this is a very worthwhile project outline and can be of great value if the results are meaningful. My only reservation is one of procedure:

For example, isolation-induced aggression encompasses two phenomena: isolation-irritability with aggressive encounters. The two are compounded when the final analysis is done: whether it is a biochemical or behavioral analysis. Additionally, these two are further compounded by nicotine treatment. Personally, I would have liked to have seen the manner in which they intend to interpret the results: this means that I would have liked to have seen the various control groups for each of the treatments and the extrapolation of an effect when comparison is made with the experimental groups. Otherwise this whole thing can end up as data salad.

Indeed, from what I know of these investigators, they are certainly competent, but sometimes even the best of us err."

I know both Volzelli and Mussini as well as the Director of the Mario Negri Institute (S. Garrattini) and think highly of all three. Unfortunately, they do not specify strains of mice or rats, which is a definite deficiency as many behavioral and biochemical differences exist among different strains.

HM:tg

1003538931

QUEENS COLLEGE

of THE CITY UNIVERSITY OF NEW YORK

FLUSHING • NEW YORK 11367

January 12, 1973

Dr. Frederic W. Nordsiek
Associate Scientific Director
The Council for Tobacco Research -
U.S.A., Inc.
110 East 59th Street
New York, New York 10022

Dear Dr. Nordsiek:

I have received your letter of January 10 and the enclosed grant application submitted to C.T.R. by Professor Luigi Valzelli. I have read the grant application very carefully and will try to provide some comments which may be useful to you in its evaluation.

The general objective of this proposal is one which I believe to be highly meritorious inasmuch as it encompasses a very important and often neglected series of variables that appear more and more to be highly relevant to both pharmacology as well as to physiological mechanisms underlying behavior. The basic model which Professor Valzelli describes is one which he has had extensive experience in developing, and his previous research on the "isolation syndrome" has suggested that principles of biochemical pharmacology are highly relevant to this model in that it represents one means by which presumed mechanisms underlying centrally-acting drugs may be investigated. The interrelationship between behavioral, pharmacological, and biochemical parameters affected by nicotine appears to me to be a not only useful but very important area for investigation. Having at his disposal a well-studied and quantifiable measure, such as aggressive behavior, enables Professor Valzelli to systematically carry out the objectives which he has specified.

Differences in the effect of nicotine and the potential relationship between differences in action based upon environmentally conferred biochemical differences in animals and their relationship in man is an exciting notion which does seem to deserve attention. In going through each of the eight areas within which Professor Valzelli has proposed examining the effects of nicotine, generally with regard to differences based upon variables related to differential housing, each appears to be a logical succession of experiments leading to some very interesting areas of investigation. Those which appear

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January 12, 1973

particularly interesting and which, to my knowledge, have certainly not been dealt with previously concern differences between the active levels of nicotine and their presumed relationship to normal and aggressive behavior. The effects of nicotine upon aggressive behavior are, of course, in themselves highly interesting in that the central effects of compounds, particularly alkaloids, which have in laboratory investigation been shown to alter aggressive behavior also usually involve changes not only in the biogenic amines, but also several other important substrates of brain metabolism. This is particularly important in the case of several of the benzodiazepines, which have been shown to alter the aggressive behavior induced by a variety of experimental techniques. Professor Valzelli's work in this area also is well-known, and I think it is particularly appropriate that he has indicated a consideration of brain N-acetyl-aspartic acid levels since these are apparently altered in animals in which experimentally-induced aggressive behavior has been manifested and such alterations are modified by anti-aggressive drugs.

A final aspect of this proposal which I think deserves some comment is the suggestion that the central effects of nicotine be considered in the partial absence of contributory aminergic nerve endings and that this paradigm be combined for aggressive and non-aggressive rats. I believe that, experimentally, this would represent a unique approach to the specification of biochemical contributions to both the effect of nicotine as well as the interaction of nicotine with those mechanisms responsible for the mediation of aggressive behavior. Personally, I like this approach very much and believe that it fits in very consistently with current methodology in neuropharmacology.

I have no question whatsoever as to the adequacy or validity of the procedures that Professor Valzelli has outlined to carry out this work. I think certainly that his well-known publications in this area and the practiced use of these techniques as have been indicated in his prior publications speak most favorably for both his competence as a scientist and his ability to carry out the proposed research.

In general, my appraisal of this proposal leads me to a highly favorable opinion of both the scientific merit as well as the importance of those contributions that could result from the proposed experiments. I believe that it certainly deserves a high level of priority.

I hope that these comments are useful to you; and if you believe that any of the other materials relevant to this proposal mentioned in

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Dr. Frederic W. Nordsiek

-3-

January 12, 1973

your letter may be helpful in further evaluation, I shall be very pleased to look at these as well.

Thank you for giving me the opportunity to read and evaluate this proposal.

With best wishes,

Sincerely,

Walter B. Essman/ss

Walter B. Essman, M.D., Ph.D.
Professor

WBE:ss

1003538934

Form:

Dr. Cattell

Dr. Bing

Dr. Jacobson

PHARMACOLOGY

No. 894

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 50TH STREET
NEW YORK, N. Y. 10022

DEC 15 1972

Application For Research Grant

Date: December 5, 1972

1. Name of Investigator(s) (include Title and Degrees)

Luigi VALZELLI, Prof., M.D., Chief Section Neuropsychopharmacol. (Director of project,
Emilio MUSSINI, Prof., M.D., Chief Section Biochem. Pharmacol. Principal Investigator)

2. Institution &

Address:

Istituto di Ricerche Farmacologiche Mario Negri, Via Eritrea 62 - 20157 Milano, Italy

3. Short Title of Project:

NICOTINE EFFECTS UPON AGGRESSIVE BEHAVIOR

4. Proposed Starting Date: July 1, 1973

5. Anticipated Duration of this Specific Study: 2 years

6. Brief Description of Objectives or Specific Aims:

Prolonged isolation in mice has been widely described as a means for inducing strongly aggressive behavior. This experimental situation was recently defined as the "isolation syndrome" because such animals show several neurochemical as well as behavioral alterations and a difference in response to many centrally acting drugs as compared with normal animals. Preliminary experiments have indicated a differential effect of nicotine in aggressive versus non-aggressive mice, (i.e., attenuation of aggressive behavior in "aggressive mice" and induction of hyperresponsivity and "aggressive" behavior in previously "non-aggressive" animals). The animals which show an experimentally-induced "isolation syndrome" show impaired learning ability and learning disturbances and also show alterations in such behavior when treated with Nicotine. The specific aims of the present research concern experiments in which the pharmacological, biochemical and behavioral parameters of Nicotine-altered behavior interactions are explored. Specially the experiments will be addressed to the issue of defining Nicotine-related changes in the biochemical modifications associated with experimentally-induced abnormalities of behavior. The correlated changes in quantified aggressive behavior would, of course, represent an integral portion of such studies.

7. Give a Brief Statement of your Working Hypothesis:

It is expected that nicotine would act differentially in normal and isolated animals due to differences in the emotional baseline upon which it acts, and which may involve differences in metabolic rate and brain level of this drug. Such indication could also be relevant for a better understanding of the activity of Nicotine in healthy and mentally disturbed men.

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8. Details of Experimental Design and Procedures: (Attach Separate Pages)

SEE ENCLOSURE A

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

SEE ENCLOSURE B

10. Additional Requirements:

Biographical sketches of all principal and professional personnel (append)

SEE ENCLOSURES C AND D

12. List of publications: (Five most recent as pertinent) (append)

SEE ENCLOSURE E and reprints attached.

1003538936

13. Budget (1st year)

\$21,748.00

A. Salaries (Personnel by names)

Professional

L. VALZELLI (Dir. of Project, Principal Invest.)

25

E. MUSSINI (Research Associate)

25

(1) Research Assistant

25

(1) Secretary

25

REDACTED

Technical

(2) Technicians

50 each

R

Sub-Total

13,000.00

B. Consumable Supplies (list by categories)

13,000.00

Animals: cost of purchasing and care

1,500.00

Glassware, Chemicals, Reagents and Drugs

800.00

Sub-Total

2,300.00

C. Other Expenses (itemize)

15,300.00

1 or 2 trips a year to U.S.A. for Federation Meetings (April) and Soc. Neurosciences Meeting (October)

1,500.00

Local travel for National and European conferences and congresses

500.00

Sub-Total

2,000.00

D. Permanent Equipment (itemize)

17,300.00

(1) Shuttle-box for rats (Basile, Italy)

433.00

(1) Animex S for animal spontaneous locomotor activity (Farad, Sweden)

2,420.00

2,853.00

E. Overhead (15% of A+B+C)

20,153.00

1,595.00

2595

Total

21,748.00

22,748

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	R	2,500.00	2,000.00		1,625.00	19,125.00
Year 3						

Signature

Director of Project

Signature

Business Officer of the Institution

Telephone

Telephone

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

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Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current

Title of Project

Source

Amount

Duration

Pending

1003538938

8. DETAILS OF EXPERIMENTAL DESIGN

Among the manifold aspects of the "isolation syndrome" in rodents, there are at least three main behavioral alterations; these consist of a) aggressive behavior, b) alterations in the learning and memory process with impairment, and c) reduction in exploratory behavior.

Moreover, isolated animals develop several differences in brain neurochemistry, principally involving brain serotonin turnover and brain N-acetyl-aspartic acid content, which may to some extent account for the alterations in behavior induced by prolonged isolation.

Based upon a series of previous experimental findings indicating that the response of such animals to centrally acting drugs is profoundly modified, as compared with normal, a series of experiments are proposed wherein the effects of nicotine are considered with regard to:

- A) general behavior of normal and isolated mice;
- B) aggressive behavior;
- C) learning and memory processes in isolated versus normal mice;
- D) exploratory behavior of isolated-aggressive mice;
- E) spontaneous locomotor activity of normal and aggressive mice;
- F) levels and turnover of brain monoamines (serotonin, norepinephrine, dopamine) in normal and isolated-aggressive mice;
- G) brain N-acetyl-aspartic acid levels;
- H) the relationship between nicotine levels (and possibly of its metabolites) in the brain of normal and aggressive animals.

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In order to determine the extent to which the monoaminergic pathways may be selectively involved in the central effects of nicotine, the activity of this drug will be studied both in normal and isolated aggressive mice previously subjected to stereotaxically or chemically placed lesions of the serotonergic or catecholaminergic systems.

The above designed experiments will be performed also in normal or isolated rats.

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PROCEDURES

- 1) Exploratory behavior in mice = hole-board test (Boissier J.R., Simon P., Lwoff J.M., *Thérapie*, 19, 571, 1964; Valzelli L., *Psychopharmacologia*, 15, 232, 1969).
- 2) Avoidance learning = shuttle-box.
- 3) Aggressive behavior = by prolonged isolation according to the method of Yen et al. (Yen C.Y., Stanger R.L., Millman N., *Arch. int. Pharmacodyn.* 123, 179, 1959), scored by Valzelli (Valzelli L., *Advances in Pharmacology*, vol. 5, pp. 79-108, 1967).
- 4) Spontaneous locomotor activity = measured by an automatized apparatus, ANIMEX S (FARAD, Sweden)
- 5) Brain monoamine extraction:
 - a) norepinephrine (NE) and dopamine (DA) according to the method described by Shore and Olin (*J. Pharmacol. exp. Ther.* 122, 295, 1958) modified by Chang (*Int. J. Neuropharmac.* 3, 643, 1964) and by Laverty and Taylor (*Anal. Biochem.* 22, 269, 1968).
 - b) serotonin (5-hydroxytryptamine = 5HT) and 5-hydroxyindoleacetic acid (5HIAA) simultaneously determined in the same brain tissue sample, according to Giacalone and Valzelli (*Pharmacology* 2, 171, 1969).
- 6) Brain monoamines detection and measurement = by an Aminco Bowman spectro-photofluorometric apparatus.
- 7) Brain amonoamine turnover calculated according to Tozer et al. (Tozer T.N., Neff N.H., Bordie B.B., *J. Pharmac. exp. Ther.* 153, 177, 1966).
- 8) N-acetyl-aspartic acid = gas chromatographically determined according to Marcucci and Mussini (*J. Chromatogr.* 25, 11, 1966).
- 9) Brain nicotine and its metabolite levels = by gas chromatographic micro-methods.
- 10) Lesions and stimulations of monoaminergic pathways = stereotaxically performed according to Dahlström and Fuxe (*Acta Physiol. Scand.* 64, suppl. 247, 1965) using the coordinates given by König and Klippel (*The rat brain, a stereotaxic atlas of the forebrain and lower parts of the brain*, Williams and Wilkins, Baltimore, 1963).
- 11) Chemical lesions = by 6-hydroxydopamine intraventricularly injected according to Noble et al. (Noble E.P., Kurtman R.J., Axelrod J., *Life Sci.* 6, 281, 1967) for the catecholaminergic system, and by 5,6-hydroxytryptamine intraventricularly injected according to Haley and McCornick (*Br. J. Pharmac.* 12, 12, 1957) for the serotonergic system.

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9. PHYSICAL FACILITIES AVAILABLE

American Instrument Co. Mod. Aminco-Bowman spectrofluorometers with Moseley Mod. Autograf recorder; Basile automatic conditioned reflex apparatus (shuttle-box); Stoelting stereotaxic apparatus for rats adaptable to mice; Beckman GMBH Mod. DU-G-2400 spectrophotometer; Elvi Mod 670 spectrophotometer; Tektronix Iscilloscope Mod. 502A; Automatized hole-board for exploratory activity (LP Italiana); Grass S-4 electronic stimulator; Beckman Mod 151 microspectrophotometer, Technicon autoanalyzer; Beckman-Spinco Mod. L. ultracentrifuge (50,000 rpm); Ivan Sorvall super-centrifuge; Carlo Erba Fractovap Mod C. gas chromatography units and one with electron capture detector; Carlo Erba Fractovap Mod. G-B gas chromatography unit; Radiometer Mod. Tritator with Tritigraph LKB RadiRac, column chromatography apparatus. Gibertini balances; thermostatic baths, shakers, mechanical and magnetic mixers, centrifuges, Basile recording thermometers, automatic syringes, Autospense Warner Chilcott automatic pipettors, calculators, homogenizers,; Reichert, Galileo, Leitz and Zeiss microscopes; Elvi and Beckman pH meters; Olivetti Program 101, desk computer.

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ENCLOSURE (C)

1003538943

Prof. LUIGI VALZELLI

CURRICULUM VITAE

October 1972

1. BIOGRAPHICAL SKETCHES

Name = VALZELLI Luigi

Present Position = Chief, Section of Neuropsychopharmacology and
Psychophysiology of the Institute for Pharmacological
Research "Mario Negri", Milano, Italy

REDACTED

Student, Faculty of Medicine and Surgery, University of
Milan

Internal student, Institute of Normal Human Anatomy and
Histology

Internal student, Institute of Pathological Human Anatomy
and Histology

Internal student, Institute of Clinical Medicine

November 17th, graduated in Medicine and Surgery (M.D.)

1952-1953 = Military services

Present military position = Captain of Sanitary Service
(in discharge)

1952-1954 = Assistant at the First-Aid Ward of the Milan Main General
Hospital (Ospedale Maggiore)

= Assistant of the Medical Division "Carati" of the Milan Main
General Hospital (Ospedale Maggiore)

1954-1955 = Assistant at the Cardiology Section of the Milan Municipality

1954-1956 = Assistant at the Neuropsychiatric Division "Origgi" of the
Milan Main General Hospital (Ospedale Maggiore)

1956 = Specialization in Cardiology, University of Pavia

= Assistant at the Institute of Pharmacology of the University
of Milan

1958 = Professoral postgraduation (Libera Docenza) in Pharmacology

= Assistant Professor of the Institute of Pharmacology of
the University of Milan

1963 = till now: Chief, Section of Neuropsychopharmacology and
Psychophysiology of the Institute for Pharmacological
Research "Mario Negri" of Milan (via Eritrea 62, 20157 Milano,
Italy)

1968 = till now: Teacher Professor of Psychophysiology and Psycho-
pharmacology of the School of Specialization in Psychology
of the University of Milan

1971 = till now: Adjunct Professor of Psychology at the Queens College
of the City University of New York

1971-1972 = Adjunct Professor at the University of Turin for the teaching
of Psychophysiology and Psychopharmacology.

He speaks and writes English, French and Spanish.

Quoted = in WHO WHO'S IN EUROPE (since 1966; 2nd Edition)

in INTERNATIONAL DIRECTORY OF INVESTIGATORS IN PSYCHOPHARMACOLOGY
(1972; p. 199)

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2. TEACHING ACTIVITIES

- 1960-1963 = Course of lessons on the Pharmacology of the Central Nervous System at the Medical Faculty of the University of Milan
- 1963 = (September); Member of the Teaching Staff of the 1st International Course on Spectrofluorimetry (held at the Institute for Pharmacological Research "Mario Negri" - Milan)
- 1965-1970 = Annual Course in Pharmacology for the students of the "Scuola per Laboratoristi Biologi" (held at the Institute for Pharmacological Research "Mario Negri" - Milan)
- 1966 = (March); Series of lessons during the 1st Course of Psychopharmacology for Practitioners (held at the Institute for Pharmacological Research "Mario Negri" - Milan)
- = (September); Lesson during the 1st Course of Instrumentation for Laboratories of BioMedical Research (held at the Institute for Pharmacological Research "Mario Negri" - Milan)
- 1968 = (February); Lesson during the 2nd Course of Instrumentation for Laboratories of BioMedical Research (held at the Institute for Pharmacological Research "Mario Negri" - Milan)
- = (March); Series of Lessons during the 2nd Course of Neuropsychopharmacology for Practitioners (held at the Institute for Pharmacological Research "Mario Negri" - Milan)
- 1968-1971 = Annual Course on Psychophysiology for the Postgraduated in Psychology of the University of Milan
- 1971 = (March); Visiting Professor at the Department of Psychology at the Queens College of the City University of New York
- = (May); Lesson at the Institute of Pharmacology of the University of Milan
- = (June); Lesson during the "Corso di aggiornamento in Neuropsicofarmacologia", held by Società Italiana di Neuropsicofarmacologia, Milan
- 1972 = (January); Lesson during the Course organized by the European Training Program in Brain and Behaviour Research at Zuoz on "Transmission and behaviour"
- = (February); Lesson at Centro di Pronto Intervento Neuropsichiatrico "R. Bozzi", Milan
- = (March) Lesson at the Institute of Veterinary of the Faculty of Medicine of the University of Milan
- 1972-1973 = (April) Course of lessons in Experimental Psychology at the Department of Advanced Psychology of the Queens College of the City University of New York
- 1972 = (June); Lesson at the Psychiatric Clinic, University of Turin.

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3. CONGRESSES

1956 = (speaker) 20th International Congress of Physiology, Bruxelles

1957 = (speaker) International Congress on "Psychotropic Drugs", Milan

1959 = (speaker) Symposium on "Pharmaka mit Wirkung auf den Monoaminstoffwechsel des Zentralnervensystem", Bad Nauheim

1960 = (speaker) Symposium on "Sindromi Depressive", Rapallo

(speaker) Symposium on "Spettrofotofluorimetria", Milan

1961 = (speaker) Symposium on "Monoamines et Systhème Nerveux Centrale", Genève

1963 = (speaker) 1st International Course on Spectrophotofluorimetry, Milan

1966 = (speaker) 5th International C.I.N.P. Symposium, Washington

(speaker) International Symposium on "Antidepressant Drugs", Milan

(speaker) Winter Meeting of the American Society of Zoologist, Washington

1967 = (speaker) International Symposium on "Biological role of Indolealkylamine derivatives", New York

(speaker) XIV Congresso Nazionale della Società Italiana di Farmacologia, Trieste

1968 = (speaker) International Symposium on "Quimioterapia de larga duracion en psiquiatria", Sitges (Barcelona)

(speaker) 6th International C.I.N.P. Symposium, Tarragona

(speaker-organizer) 1st International Congress on "Aggressive Behavior", Milan

(speaker) International Meeting of the Italian and British Societies of Pharmacology, Florence

(speaker) Table Ronde sur la Sérotonine et Pathologie Digestive, Vichy

(speaker) 1st International Congress of the Collegium Internationale Activitas Nervosae Superioris (C.I.N.S.), Milan

1969 = (chairman) International Symposium on "Amphetamines and related compounds", Milan

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(speaker) II Riunione Nazionale della Società Italiana di Neuropsicofarmacologia, Tirrenia (Pisa)

(speaker) IV International Congress on Pharmacology, Basel

(organizer-chairman) Round Table on "Correlation between behaviour and transmitters (suspects) in brain", during the 2nd International Meeting of the International Society for Neurochemistry, Milan

(speaker) Round Table on "Serotonina in Patologia Umana", Fondazione C. Erba, Milan

(speaker) XV Congress of the Società Italiana di Farmacologia, Milan

(speaker) 2nd International Congress of the Polish Pharmacological Society, Warsaw

(speaker) 1st Interdisciplinary Meeting of Psychobiology, Parma

(organizer) 2nd Interdisciplinary Meeting of Psychobiology, Milan

(speaker) Round Table on Learning, Como

1970 = (speaker) Meeting of Gerontology, Milan

(speaker) Round Table on "Ipnosi, mezzi farmacologici e libertà individuale", Fondazione C. Erba, Milan

(speaker) Round Table on "Orientamenti attuali sul trattamento della malattia di Parkinson", Fondazione C. Erba, Milan

(speaker) Round Table on "Psychotomimetic drugs", Varese

(speaker) Round Table on "I giovani e la droga", Fondazione C. Erba, Milan

(speaker) Congress on "Atherosclerosis and Central Nervous System", Fondazione C. Erba, Milan

(speaker) 3rd Interdisciplinary Meeting on Psychobiology on "Genetica e ambiente nel determinismo del comportamento degli animali e dell'uomo", Pavia

(speaker) International Symposium on "Migraine and headache", Florence

(speaker) Congress on "Malformazioni, tumori, difetti mentali e correlazioni patogenetiche", Fondazione C. Erba, Milan

(speaker) 3rd Meeting of the Società Italiana di Neuropsicofarmacologia on "Farmacoterapia della schizofrenia", Milan

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- (speaker) Round Table on Psychotomimetic Drugs, Gallarate
- (speaker) Meeting on Neurobiology, Rome
- (chairman) NATO Course on Chemistry of Brain Development, Milano
- (speaker) 39th Meeting of "Unione Zoologica Italiana", Salice Terme (Pavia)
- (speaker) Round Table on "Associazioni Psicofarmacologiche" Società Italiana di Neuropsicofarmacologia Castelvechio Pascoli, (Lucca)
- (speaker) Workshop of the European Training Program in Brain and Behaviour Research, Milan
- 1971 = (speaker) Meeting at Istituto Carlo Erba, Milan
- (speaker) Round Table on "Droghe e Minidroghe", Fondazione C. Erba, Milan
- (Honor guest) Congress on "Monoamineoxidases: New Vistas" Cagliari
- (chairman) Congress on "Physiology and Pharmacology of Cyclic AMP", Milan
- (speaker-chairman) Joint Meeting of the Italian Pharmacological Society and the Belgian Physiological and Pharmacological Society, Gent - Belgium.
- (speaker) Round Table on "Antiaggression" during the "International Symposium on Benzodiazepines", Milan
- 1972 = (speaker) Round Table on "Crisi dei giovani, droga, educazione" Fondazione C. Erba, Milan
- (speaker) Federation Meetings, Atlantic City
- (speaker) Neurophysiology Group Meeting on "Brain lesions and drug action", Atlantic City
- (speaker) Workshop on Recent Advances in the "Psychobiology of Electroconvulsive Therapy", Puerto Rico
- (speaker) Meeting on Learning and Memory, Ministero Pubblica Istruzione, Como
- (chairman) Corso di aggiornamento in neuropsicofarmacologia on "Farmaci attivi sulle insonnie", Milan
- (speaker) International Symposium on "Metabolic regulation and functional activity in the Central Nervous System" Saint Vincent
- (speaker) Meeting on "Recenti sviluppi nella psicologia dell'apprendimento", Frascati

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4. LECTURES

- 1967 = (April); "Biochemical aspects and clinical implications of aggressive behaviour"; Endo Laboratories Inc., Garden City, New York
- (May); "Comportamiento agresivo: fechas bioquímicas y farmacológicas"; Institute of Pharmacology, University of Barcelona
- (November); "Biochemical and behavioural aspects of aggressiveness"; J.R. Geigy S.A., Basel
- (December); "L'aggressività sperimentale", Psychiatric Clinic of the University of Genoa
- 1968 = (March); During the 2nd Course of Neuropsychopharmacology for Practitioners at the Institute for Pharmacological Research "Mario Negri", Milan
- (April); "Antidepressant drugs", Institute of Pharmacology, University of Cagliari
- (November); "Experimental aggressiveness: psychological, methodological, biochemical and pharmacological aspects"; Department of Pharmacology, Karolinska Institutet of Stockholm
- 1969 = (October); "Some aspects of induced aggressiveness in mice and rats" Department of Pharmacology, Medical Academy of Warsaw
- 1970 = (June); "Anticipazione dell'apprendimento e strutture cerebrali"; Ministero della Pubblica Istruzione
- (November); "Nuove correnti scientifiche di trattamento medico-pedagogico del minorato" Società Italiana per l'Assistenza Medico-Psico-Pedagogica ai minorati dell'età evolutiva (SIAME), Sanremo
- (November); "Memory mechanisms"; Clinica delle Malattie Nervose e Mentali, University of Rome
- (November); "Memory mechanisms"; Clinica delle Malattie Nervose e Mentali, University of Siena
- (November); "Sperimentazione e ricerche comprovanti il valore di alcuni fattori indispensabili allo sviluppo delle capacità di apprendimento"; Ministero della Pubblica Istruzione, Luino
- (November); "Il problema del sonno"; Clinica Psichiatrica dell'Università di Verona
- (December); "Aspetti psicosociali della droga"; PRO-FAR-BER, Bergamo

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1971 = (March); "Intéraction entre état émotif et activité des substances psychoactives" Unité de Neuropharmacologie, Paris

(March); "Agressivité chez le rat et la souris: aspects comportementaux et biochimiques"; Medical Faculty of Paris, (Actualités pharmacologiques)

(March); "Lesion and stimulation of brain structures as a tool to study mechanisms of drug action"; National Institutes of Mental Health, Saint Elizabeth's Hospital, William A. White Building, Washington D.C.

(April); "Neurochemistry of the Central Nervous System"; Centro di Pronto Intervento Neuropsichiatrico "R. Bozzi", Milan

(April); "Neurochemical correlates of behaviour"; Centro di Pronto Intervento Neuropsichiatrico "R. Bozzi", Milan

(May); "Substrato emotivo come condizionante l'attività degli psicofarmaci"; Clinica Psichiatrica dell'Università di Pavia

(October); "Aggressive behaviour: psychophysiological substrates, environmental influences, neurochemical aspects"; Clinica Psichiatrica, Università di Genova

1972 = (March); Seminar on "Aspetti psicosociali della droga" at Lions' Club of Mariano-Comense (Como, Italy)

(April); "Some biochemical and behavioral correlates of induced aggressiveness"; Columbia University, New York

(April); "Aggressiveness by isolation in mice and rats: behavioral and pharmacological aspects"; Temple University, Department of Pharmacology, Philadelphia.

(April); "Aggressiveness by isolation and drug effects: some biochemical and behavioral correlates of induced aggression"; Shering Corporation, Bloomfield, N.J.

(April); "Behavioral and neurochemical aspects of aggressiveness"; University of Connecticut, Dpt. Biobehavioral sciences, Storrs

(April); "Emotional modulation of psychotropic drugs"; University of Connecticut, Dpt. Biobehavioral sciences, Storrs

(May); "Lésions ou stimulations des voies monoaminergiques centrales et activité de quelques substances"; Centre National de la Recherche Scientifique, Centre de Neurochimie, Strasbourg

(May); "Aspect comportementaux, biochimiques et pharmacologiques de l'agressivité par isolement dans la souris et le rat"; Centre National de la Recherche Scientifique, Centre de Neurochimie, Strasbourg

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5. RESEARCH GRANTS

- 1963 = "Isolation of physiological active substances from brain"
U.S.A. Army contract n. DA-91-591-EUC-2687 - with S. Garattini
- 1964 = "Isolation of physiological active substances from brain"
U.S.A. Army contract n. DA-91-591-EUC-3234 - with S. Garattini
- 1966 = "Pharmacological and biochemical changes in animals made aggressive by isolation"
U.S.A. Army contract n. DA-91-591-EUC-4058 - with S. Garattini
- 1967 = "Pharmacological and biochemical changes in animals made aggressive by isolation"
U.S.A. Army contract n. DAJA-37-67-C-0586 - with S. Garattini
- = "Effect of drugs on age and behavior"
National Institutes of Health, Bethesda, Md., U.S.A., contract n. DHEW/PHS/NIH PH-43-67-83 - with S. Garattini
- 1968 = "Pharmacological and biochemical changes in animals made aggressive by isolation"
U.S.A. Army contract n. DAJA-37-68-C-1076 - with S. Garattini
- = "Effect of drugs on age and behavior"
National Institutes of Health, Bethesda, Md., U.S.A., contract n. DHEW/PHS/NIH PH-43-67-83 - with S. Garattini
- 1969 = "Effect of drugs on age and behavior"
National Institutes of Health, Bethesda, Md., U.S.A., contract n. DHEW/PHS/NIH PH-43-67-83 - with S. Garattini
- "Activité du Sulpiride (Dogmatil) sur les activités nerveuses supérieures"
Laboratoires Delagrangé, Paris
- 1970 = "Activité du Sulpiride (Dogmatil) sur les activités nerveuses supérieures"
Laboratoires Delagrangé, Paris
- "Aspetti farmacologici e biochimici della aggressività sperimentale da isolamento"
C.N.R. (Centro Nazionale della Ricerca, Roma), contract n. 70.01143.04
- 1971 = "Aspetti farmacologici e biochimici della aggressività sperimentale da isolamento"
C.N.R. (Centro Nazionale della Ricerca, Roma), contract n. 70.01143.04

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1971 = "Attività della Caffaina sul Sistema Nervoso Centrale"
Crippa & Berger, Milan.

1971-1972= "Study on the comparative pharmacology of steroid contraceptive
drugs"
National Institute of Child Health and Human Development, U.S.A.

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6. MEMBERSHIPS

REDACTED

Correspondent Member of the Journal "Bulletin de Recherches Thérapeu-
tiques".

Referee for Psychopharmacology of the "European Journal of Pharmacology",
and "Pharmacological Research Communications".

7. AWARDS

1968 = "Ambrogino d'oro" from the Comune of Milano for the contri-
bution as organizer of the 1st International Congress on
"Aggressive Behaviour" (Milan, 1968).

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8. BOOKS

- 1965 = "Serotonin", with S. Garattini
Elsevier Publishing Company, Amsterdam
- 1967 = contributor to "Advances in Pharmacology", vol.5, Academic
Press Inc., New York
- 1970 = contributor with S. Garattini to the volume "Principles
of Psychopharmacology" (W.G. Clark and J. Del Giudice, eds.)
Academic Press Inc., New York
- italian translation of the volume "Clinical Psychopharmacology"
By M. Shepherd, M. Lader and R. Rodnight (C. Manfredi, Milan)
- "Elementi di Psicofarmacologia, Sperimentale e Clinica"
C. Manfredi, Milan
- 1971 = "Profili di Psicofisiologia e Neurochimica"
C. Manfredi, Milan
- 1973 = "Psychopharmacology: an introduction to experimental and clinical
principles"
Spectrum Publications, New York, W.B. Essman editor.

9. PUBLICATIONS

October 1972 = 120 published papers and 8 papers in press.

See the enclosed list.

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ELENCO DEI
LAVORI PUBBLICATI

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tonin after electrical shock"

L. Valzelli (in collaborazione con S. Garattini e A. Valsecchi)

Experientia, 13 (1957) 330

18 "Serotonin and electroshock"

— L. Valzelli (in collaborazione con S. Garattini)

Atti del Congresso Internazionale sui Farmaci Psicotropi (Milano, 1957)

Eds. S. Garattini & V. Ghetti - Elsevier Publ. Co., Amsterdam, pag. 435

19 "Azione della veratrina sul contenuto in serotonina del cervello e dell'intestino di ratto"

L. Valzelli (in collaborazione con A. Bertelli)

Atti Soc. Lomb. Sci. Med. Biol., 13 (1958) 156

20 "Ancora sui rapporti tra elettroshock e serpina"

L. Valzelli (in collaborazione con R. Kato e L. Lanesta)

Boll. Soc. It. Biol. Sper., 34 (1958) 248

21 "Researches on the mechanism of the reserpine sedative action"

— L. Valzelli (in collaborazione con S. Garattini)

Science, 123 (1953) 1278

22 "Sul contenuto in serotonina del polmone dopo shock anafilattico"

— L. Valzelli (in collaborazione con L. Ma-

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riani)

Boll.Soc.It.Biol.Sper., 34 (1958) 1169

- 23 "Serotonina e anuria sperimentale del rat
to"

L. Valzelli (in collaborazione con L. Ma-
riani)

Boll.Soc.It.Biol.Sper., 34 (1958) 1167

- 24 "Effetto precipitante del rame su sieri
provenienti da diverse specie animali"

L. Valzelli (in collaborazione con E. Mus-
sini)

Boll.Soc.It.Biol.Sper., 34 (1958) 1483

- 25 "Aumento di tossicità della 5-idrossitrip-
tamina ad opera della iproniazide"

L. Valzelli (in collaborazione con B. Bil-
la)

Boll.Soc.It.Biol.Sper., 34 (1958) 1404

- 26 "Sul contenuto in 5-idrossitriptamina in
diverse aree cerebrali del cane dopo elet-
troshock"

L. Valzelli (in collaborazione con P. Fre-
sia, E. Genovese e R. Kato)

Boll.Soc.It.Biol.Sper., 34 (1958) 1397

- 27 "Cortisone e 5-idrossitriptamina cerebra-
le"

L. Valzelli (in collaborazione con R. Ka-
to)

Boll.Soc.It.Biol.Sper., 34 (1958) 1402

- 28 "La clorpromazina inibisce la fissazione

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- di serotonina al tessuto polmonare"
L. Valzelli (in collaborazione con R. Kato e L. Mariani)
Atti Soc.Lomb.Sci.Med.Biol., 13 (1958) 297
- 29 "Ancora sui rapporti tra convulsioni e serotonina cerebrale"
— L. Valzelli (in collaborazione con S. Garattini e R. Kato)
Atti Soc.Lomb.Sci.Med.Biol., 13 (1958) 300
- 30 "L'aumento di serotonina cerebrale dopo elettroshock non è in rapporto con lo stato convulsivo"
— L. Valzelli (in collaborazione con M. Bissiani, S. Garattini e R. Kato)
Atti Soc.Lomb.Sci.Med.Biol., 13 (1958) 345
- 31 "Sostanze indoliche presenti nelle banane e modificazioni nel ricambio indolico in rapporto con dieta a base di banane"
— L. Valzelli (in collaborazione con E. Musini)
Atti Soc.Lomb.Sci.Med.Biol., 13 (1958) 310
- 32 "Effetto della metionina sulle proteine del siero in diverse condizioni sperimentali di danno epatico"
— L. Valzelli (in collaborazione con V. Palma e A. Vegeto)
Atti Soc.Lomb.Sci.Med.Biol., 13 (1958) 453
- 33 "Reserpine derivatives with specific hypotensive or sedative activity"
— L. Valzelli (in collaborazione con S. Ga-

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rattini, A. Mortari e A. Valsecchi)
Nature, 183 (1959) 1273

34 "Funzione tiroidea e serotonina encefalica"
L. Valzelli (in collaborazione con R. Kato)
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ENCLOSURE

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(4)

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CURRICULUM VITAE

October 1972

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✓ 1954 = Fellowship, Pasteur Institute, Paris

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✓ 1959 = Professoral Postgraduation (Libera Docenza) in Pharmacology

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✓ 1966 = Visiting Scientist, National Institutes of Health, Bethesda, Md.,
Section of Clinical Biochemistry

1970 = Member of the Council for Biomedical Technology of the "Centro Nazionale delle Ricerche" (C.N.R.).

He speaks and writes English and French.

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2. TEACHING ACTIVITY

- 1955-1963 = Courses of Lessons for Medical Students at the Medical Faculty of the University of Milan.
- 1963 = (September); Member of the teaching staff of the 1st International Course on Spectrofluorometry (held by the Istituto di Ricerche Farmacologiche "Mario Negri", Milan)
- 1966 = (March); Member of the teaching staff of the 1st Course of Psychopharmacology for Practitioners (held by the Istituto di Ricerche Farmacologiche "Mario Negri", Milan)
- = (September); Lesson during the 1st Course of Instrumentation for Laboratories of BioMedical Research (held by the Istituto di Ricerche Farmacologiche "Mario Negri", Milan)
- 1968 = (February); Lesson during the 2nd Course of Instrumentation for Laboratories of BioMedical Research (held by the Istituto di Ricerche Farmacologiche "Mario Negri", Milan)
- = (March); Member of the teaching staff of the 2nd Course of Neuropsychopharmacology for Practitioners (held by the Istituto di Ricerche Farmacologiche "Mario Negri", Milan)
- 1970 = (May) Lesson on Gas Chromatography at the Course on Instrumentation for Laboratories of BioMedical Research (held by the Istituto di Ricerche Farmacologiche "Mario Negri", Milan).

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3. CONGRESSES

- 1963 = (Speaker) at the "International Course on Spectrophotofluorometric techniques in biology", Milan
- 1964 = (Speaker) at the "International Symposium on non-steroidal anti-inflammatory drugs", Milan
- 1965 = (Speaker) at the Technicon Symposium in Paris
(Speaker) at the Technicon Symposium in London
(Speaker) at the Technicon Symposium in Frankfurt
(Speaker) at the Technicon Symposium in Rome
- 1966 = (Speaker) at the 2° Symposium Mario Negri, Milan
- 1967 = (Speaker) at the 3° Symposium Mario Negri, Milan
- 1968 = (Speaker) at the 4° Symposium Mario Negri, Milan
- 1969 = (Speaker) at the 5° Symposium Mario Negri, Milan
- 1971 = (Speaker) at the "International Symposium on Benzodiazepines", Milan.

4. LECTURES

- 1961 = Lectures on "Aminoacids in the rat brain after treatment with psychotropic drugs" at City of Hope, Duarte, Calif.
- 1965 = Lectures on gas-chromatography techniques at the Symposiums held by Technicon in Paris, London, Frankfurt and Rome

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5. RESEARCH GRANTS

- 1963 = "Isolation of physiologically active substances from brain"
U.S.A. Army contract n. DA-91-591-EUC-2687 - with S. Garattini
- 1964 = "Isolation of physiologically active substances from brain"
U.S.A. Army contract n. DA-91-591-EUC-3234 - with S. Garattini
- 1965-1969 = "A study on the mechanism of gossypol toxicity counteraction by
L-lysine, to gain information needed to permit the increased
use of cottonseed products in animal feeds"
Fg-It-131 contract, - with S. Garattini
- 1970-1971 = Programma Speciale "Tecnologie Biomediche"
C.N.R. Contract n. 70.00716/31-17-9-3 - with S. Garattini
- 1971-1972 = "Study on the comparative pharmacology of steroid contraceptive
drugs"
National Institute of Child Health and Human Development, U.S.A.,
contract n. NIH-NICDH-72-2733 - with S. Garattini
- Programma Speciale "Tecnologie Biomediche; Tossicità dei Farmaci"
C.N.R. Contract n. 70.00716/31-17-9-3 - with S. Garattini

6. MEMBERSHIPS

REDACTED

Referee of the "European Journal of Pharmacology."

7. PUBLICATIONS

October 1972 = 68 published papers and 3 papers in press (see enclosed list).

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LIST OF PUBLICATIONS

1. Garattini S., Mussini E.: Fegato in degenerazione grassa ed inibizione di sue attività a tipo disulfuramico. Atti Soc. Lomb. Sci. Med. Biol., 1953, 8, 444.
2. Bassoli G., Benati E., Mussini E.: Cortisone, ormone somatotropo, gruppi sulfidrilici del fegato e reazione cutanea alla tubercolina. Giorn. It. Chemioter., 1954, 1, 539.
3. De Poli A., Mussini E.: Titolazione amperometrica dei gruppi sulfidrilici nel cristallino catarroso. Atti Soc. Oftalmol. Lomb., 1954, II.
4. Garattini S., Mussini E.: Sulla distribuzione degli SH nell'organismo. Atti Soc. Lomb. Sci. Med. Biol., 1954, 2, 125.
5. Garattini S., Mussini E.: Ricerche sul meccanismo dell'azione diuretica dei mercuriali. Atti Soc. Lomb. Sci. Med. Biol., 1954, 2, 131.
6. Garattini S., Mussini E.: Sui rapporti tra ATP e gruppi sulfidrilici. Boll. Soc. It. Biol. Sper., 1954, 30, 1114.
7. Garattini S., Mussini E.: Sul contenuto in gruppi sulfidrilici nel fegato dopo trattamento con aminoacidi solforati. Boll. Soc. It. Biol. Sper., 1954, 30, 1117.
8. Garattini S., Mussini E.: Aumento di gruppi sulfidrilici nell'ipofisi durante la gravidanza. Boll. Soc. It. Biol. Sper., 1954, 30, 1119.
9. Garattini S., Mussini E., Paoletti R.: Sulla possibilità di antagonizzare gli effetti tossici dell'isoniazide (INI). Giorn. It. Chemioter., 1954, 1, 262.
10. Bassoli G., Mussini E.: Sul comportamento delle proteine e dei gruppi sulfidrilici in rapporto con la gravità della malattia tubercolare. Giorn. It. Chemioter., 1955, 2, 83.
11. Bertelli A., Mussini E.: Azione della strofantina sui gruppi-SH del cuore. Boll. Soc. It. Biol. Sper., 1955, 31, 1168.
12. Cocucci M., Garattini S., Mussini E.: Sul comportamento di gruppi sulfidrilici del fegato dopo trattamento con cortisone, idrocortisone, metacortandracin. Boll. Soc. It. Biol. Sper., 1955, 31, 850.

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13. Ferri L., Mussini E., Palazzi D.: Gruppi sulfidrilici nel sarcoma 180 del topo e nell'adenocarcinoma di Walker del ratto dopo irradiazione. Tumori, 1955, 29, 424.
14. Garattini S., Mussini E.: Separazione cromatografica su carta di alcune purine (6-mercaptapurina, tioguanina, adenina e guanina). Boll. Soc. It. Biol. Sper., 1955, 31, 870.
15. Bizzi L., Murelli B., Mussini E.: Rame e sue diverse fissazioni al siero ed ai tessuti nel ratto e nella cavia. Arch. E. Maragliano, 1957, 13, 1.
16. Leonardi A., Mussini E.: Aminoacidi presenti nel siero dopo trattamento cortisonico in animali normali e surrenectomizzati. Giorn. It. Chemioter., 1958, 5, 179.
17. Marcucci F., Mussini E.: Diminuizione della eliminazione urinaria di taurina dopo trattamento prolungato con isoniazide. Boll. Soc. It. Biol. Sper., 1958, 34, 1103.
18. Marcucci F., Mussini E.: Diminuizione della eliminazione biliare di acido taurocolico dopo trattamento prolungato con isoniazide. Boll. Soc. It. Biol. Sper., 1958, 34, 1104.
19. Marcucci F., Mussini E.: Sulla eliminazione di acido cisteico e di taurina dopo trattamento con isoniazide. Boll. Soc. It. Biol. Sper., 1958, 34, 1422.
20. Marcucci F., Mussini E.: Metabolismo degli aminoacidi solforati in animali trattati cronicamente con idrazide isonicotinica. Giorn. It. Chemioter., 1958, 5, 183.
21. Mussini E.: Sulla distribuzione nell'organismo e sull'attività diuretica del p-cloro mercuri-benzoato. Boll. Soc. It. Biol. Sper., 1958, 34, 1586.
22. Mussini E.: Nuovo rapido metodo di dosaggio dei gruppi sulfidrilici in materiali biologici. Boll. Soc. It. Biol. Sper., 1958, 34, 1419.
23. Mussini E.: Distribuzione dei diuretici mercuriali nell'organismo. Boll. Soc. It. Biol. Sper., 1958, 34, 1487.
24. Mussini E.: Sui legami dei diuretici mercuriali alle proteine del siero. Boll. Soc. It. Biol. Sper., 1958, 34, 1588.
25. Mussini E.: Sulla distribuzione nell'organismo o sulla attività diuretica del p-cloro-mercuri-benzoato. Boll. Soc. It. Biol. Sper., 1958, 34, 1586.

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26. Mussini E.: Mancato effetto dell'isoniazide sul contenuto urinario di guanidotelaurina e carbamidolaurina. Atti Soc. Lomb. Sci. Med. Biol., 1958, 3, 212.
27. Mussini E.: Aminoacidi presenti nel siero dopo trattamento cortisonico in animali normali e surrenectomizzati. Giorn. It. Chemioter., 1958, 5, 77.
28. Mussini E., Valzelli L.: Sostanze idoliche presenti nelle banane e modificazioni nel ricambio indolico in rapporto a diete a base di banane. Atti Soc. Lomb. Sci. Med. Biol., 1958, 13, 310.
29. Mussini E., Valzelli L.: Effetto precipitante svolto dal rame su sieri provenienti da diverse specie animali. Boll. Soc. It. Biol. Sper., 1958, 34, 1438.
30. Marcucci F., Mussini E.: A new apparatus for thin-layer chromatography. J. Chromatogr., 1963, 11, 270.
31. Marcucci F., Mussini E.: Separation of proline and hydroxyproline derivatives by thin-layer chromatography. J. Chromatogr., 1965, 18, 431.
32. Marcucci F., Mussini E., Poy F., Gagliardi P.: Separation of amino acids as their N-trifluoroacetyl-n-butyl esters by gas chromatography. J. Chromatogr., 1965, 18, 487.
33. Mussini E., Marcucci F.: Separation of prolines and hydroxyprolines by gas chromatography. J. Chromatogr., 1965, 20, 266.
34. Mussini E., Marcucci F.: Separation of amino acid n-butyl esters by means of thin-layer chromatography. J. Chromatogr., 1965, 17, 576.
35. Marcucci F., Mussini E.: A method for the gas-chromatographic analysis of n-acetyl-aspartic acid in brain. J. Chromatogr., 1966, 25, 11.
36. Marcucci F., Mussini E., Valzelli L., Garattini S.: Distribution of N-acetyl-L-aspartic acid in rat brain. J. Neurochem., 1966, 13, 1069.
37. Mussini E., Hutton J.J.Jr., Udenfriend S.: Collagen proline hydroxylase in wound healing, granuloma formation, scurvy, and growth. Science, 1967, 157, 927.
38. Mussini E., Marcucci F.: The preparation of diazopropane and diazobutane for the esterification of amino acids. J. Chromatogr., 1967, 26, 481.
39. Mussini E., Marcucci F., Garattini S.: Postnatal changes in brain N-acetyl-L-aspartic acid content of normal and hypothyroid suckling rats. J. Neurochem., 1967, 14, 551.

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40. Marcucci F., Fanelli R., Mussini E.: A method for gas-chromatographic determination of benzodiazepines. J. Chromatogr., 1968, 37, 318.
41. Marcucci F., Guaitani A., Kvetina J., Mussini E., Garattini S.: Species differences in diazepam metabolism and anticonvulsant effect. Europ. J. Pharmacol., 1968, 4, 467.
42. Marcucci F., Mussini E.: A metabolic explanation for differences between species of the anticonvulsant activity of diazepam. Brit. J. Pharmacol., 1968, 34, 667P.
43. Marcucci F., Mussini E., Valzelli L., Garattini S.: Decrease in N-acetyl-L-aspartic acid in brain of aggressive mice. J. Neurochem., 1968, 15, 53.
44. Morselli P.L., Garattini S., Marcucci F., Mussini E., Rewerski W., Valzelli L., Peters R.A.: The effect of injections of fluorocitrate into the brains of rats. Biochem. Pharmacol., 1968, 17, 195.
45. Bartosch I., Mussini E., Garattini S.: Reduction of nitrazepam by rat liver. Biochem. Pharmacol., 1969, 18, 2263.
46. Garattini S., Marcucci F., Mussini E.: Gas chromatographic analysis of benzodiazepines. In "Gas chromatography in Biology and Medicine", Porter R. ed., J.A. Churchill Ltd., London, 1969, p.161.
47. Marcucci F., Airolidi L., Mussini E.: Brain level of N-acetyl-L-aspartate in different strains of mouse and rat. J. Neurochem., 1969, 16, 272.
48. Marcucci F., Fanelli R., Mussini E., Garattini S.: The metabolism of diazepam by liver microsomal enzymes of rats and mice. Europ. J. Pharmacol., 1969, 7, 307.
49. Bartosch I., Mussini E., Saronio C., Garattini S.: Studies on nitrazepam reduction "in vitro". Europ. J. Pharmacol., 1970, 11, 249.
50. Garattini S., Marcucci F., Mussini E.: Studies on the anticonvulsant action of diazepam. Brit. J. Pharmacol., 1970, 38, 455P.
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52. Marcucci F., Fanelli R., Mussini E., Garattini S.: Further studies on species difference in diazepam metabolism. Europ. J. Pharmacol., 1970, 2, 253.

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53. Marcucci F., Fanelli R., Mussini E., Garattini S.: Further studies on the long lasting antimetabolite activity of diazepam in mice. Europ. J. Pharmacol., 1970, 11, 115.
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56. Forgiione A., Martelli P., Marcucci F., Fanelli R., Mussini E., Jommi G.C.: Gas liquid chromatography and mass spectrometry of various benzodiazepines. J. Chromatogr., 1971, 59, 163.
57. Marcucci F., Airolidi L., Mussini E., Garattini S.: A method for gas chromatographic determination of butyrophhenones. J. Chromatogr., 1971, 59, 174.
58. Marcucci F., Airolidi M.L., Mussini E., Garattini S.: Brain level of metrazol determined with a new gas chromatographic procedure. Europ. J. Pharmacol., 1971, 16, 219.
59. Marcucci F., Guaitani A., Fanelli R., Mussini E., Garattini S.: Metabolism and anticonvulsant activity of diazepam in guinea pigs. Biochem. Pharmacol., 1971, 20, 1711.
60. Marcucci F., Mussini E., Airolidi L., Fanelli R., Frigerio A., De Nadai F., Bizzi A., Rizzo M., Morselli P.L., Garattini S.: Analytical and pharmacokinetic studies on butyrophhenones. Clin. Chim. Acta, 1971, 34, 321.
61. Marcucci F., Mussini E., Guaitani A., Fanelli R., Garattini S.: Anticonvulsant activity and brain levels of diazepam and its metabolites in mice. Europ. J. Pharmacol., 1971, 16, 311.
62. Mussini E., Marcucci F., Fanelli R., Garattini S.: Metabolism of diazepam and its metabolites by guinea pig liver microsomes. Biochem. Pharmacol., 1971, 20, 2529.
63. Bertagni P., Marcucci F., Mussini E., Garattini S.: Biliary excretion of conjugated hydroxyl benzodiazepines after administration of several benzodiazepines to rats, guinea pigs, and mice. J. Pharm. Sci., 1972, 61, 965.

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64. Bizzzi A., Tacconi M.T., Airolidi L., Marcucci F., Mussini E., Garattini S.: Haloperidol and trifluoperidol in rat adipose tissue. Rev. Europ. Etud. Clin. Biol., 1972, 17, 169.
65. Marcucci F., Airolidi L., Mussini E., Garattini S.: Distribution of haloperidol and trifluoperidol in brain and blood of rats. Chem.-Biol. Interactions, 1972, 4, 427.
66. Marcucci F., Mussini E., Airolidi L., Guaitani A., Garattini S.: Brain concentrations of lorazepam and oxazepam at equal degree of anticonvulsant activity. J. Pharm. Pharmacol., 1972, 24, 63.
67. Mussini E., Marcucci F., Fanelli R., Garattini S.: Metabolism of diazepam metabolites in guinea pigs. Chem.-Biol. Interactions, 1972, 2, 73.
68. Mussini E., Marcucci F., Fanelli R., Guaitani A., Garattini S.: Analytical and pharmacokinetic studies on the optic isomers of oxazepam succinate half-ester. Biochem. Pharmacol., 1972, 21, 127.
69. Garattini S., Marcucci F., Mussini E.: Benzodiazepine metabolism in vitro. Drug Metabolism Reviews, in press.
70. Garattini S., Marcucci F., Morselli P.L., Mussini E.: The Significance of measuring blood levels of benzodiazepines. In "The Benzodiazepines", Garattini S. and Randall L.O. eds., Raven Press, New York, 1973 in press.
71. Mussini E., Marcucci F., Airolidi L., Fanelli R., Morselli P.L., Garattini S.: Urinary excretion of C₃ hydroxylated benzodiazepines in man. Europ. J. Clin. Pharmacol., in press.

vp/ November 1972

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#776A VESSEL

1003538993

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 2, 1973

Grant Application No. 776A

5
Rejected

To: The committee comprising Drs. Bing, Cattell and Loosli
Subject: Elliot S. Vesell, M.D., Pennsylvania State University
College of Medicine, Hershey, Pennsylvania
Continuation application 776A
"Radioimmunoassay for Nicotine"

History

This investigator has been supported by CTR since 1971. The current grant, \$29,555, is terminal with the stated hope that development of the radioimmunoassay will have been completed.

This hope has not been fulfilled and one additional year at \$34,730 is now requested, with a stated pledge of completion. CTR has no commitment to provide this support.

Documents Submitted

We are now forwarding only the application dated 1/23/73 with supporting data attached.

(Six reprints provided show that Dr. Vesell has been co-author or author of six papers published during the last year or so. They are not forwarded as they appear not directly relevant to this project.)

Comment

Dr. Vesell has telephoned several CTR staff members urging that this request be given priority in competition (although he acknowledges that we have no commitment) so he may complete development of the assay method.

FWM
F.W.N

FWN:wg
Encl.

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Comm.

Dr. Bing
Dr. Cattell
Dr. Loosli

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 58TH STREET
NEW YORK, N. Y. 10022
(212) 421-8985

FEB 1 1973

Application for Research Grant
(Use extra pages as needed)

#776A

#776M2-R1-6/1/72-

5/31/73

#776-6/1/71-5/31/72

Date: 1/23/73

1. Principal Investigator (give title and degrees):

Elliot S. Vesell, A.B., M.D., Professor and Chairman, Dept. Pharmacology, and
Professor of Medicine and Genetics
G. Thomas Passananti, B.S., M.S., Ph.D., Asst. Professor, Dept. Pharmacology

2. Institution & address:

Pennsylvania State University College of Medicine
Milton S. Hershey Medical Center
Hershey, Pennsylvania 17033

3. Department(s) where research will be done or collaboration provided:

Pharmacology

4. Short title of study:

Radioimmunoassay for Nicotine

5. Proposed starting date: June 1, 1973

6. Estimated time to complete: One year

7. Brief description of specific research aims:

As mentioned in my application to the Council for Tobacco Research dated July 1, 1970, the objectives of this study are to "determine the rate of elimination of nicotine from the blood and urine of smokers and nonsmokers, to define the range of individual variation of nicotine metabolism in these two groups and to determine whether chronic nicotine administration causes induction of the hepatic microsomal drug-metabolizing enzymes responsible for biotransformation of nicotine." To achieve these aims, plasma levels of nicotine will have to be determined after nicotine is administered in various forms. We plan to measure rates of nicotine decay from plasma (nicotine pharmacokinetics). We are concerned with the effects of different types of environment (previous exposure to nicotine, particularly) on nicotine kinetics. These kinetic data on nicotine decay from plasma would be obtained in naive and confirmed cigarette smokers, cigar smokers and pipe smokers. Thus variations in nicotine kinetics would be examined not only within each group but also among the different groups. Hopefully, this would provide data not previously available on the role of different types of environment on rates of nicotine decay from plasma.

Such kinetic studies on nicotine metabolism have not previously been performed but short time points of blood sampling ranging from 2 minutes to 30 minutes after exposure to nicotine would be obtained initially because we anticipate a very rapid decline in nicotine blood concentrations after nicotine is absorbed. Nicotine decay from blood would be investigated in certain of these studies after the individuals inhaled several puffs of a cigarette or after 15 minutes of pipe or cigar smoking. To perform this study a rapid, extremely sensitive nicotine assay is required.

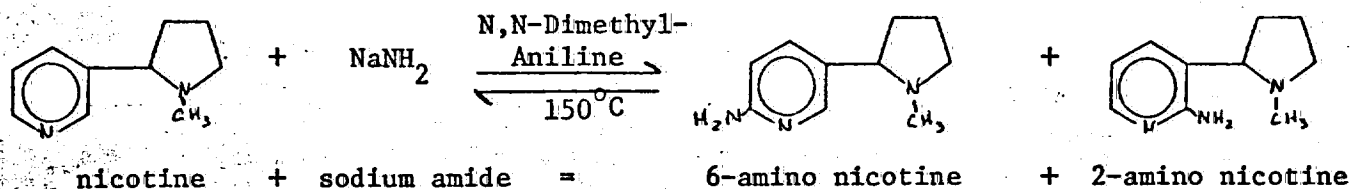
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8. Brief statement of working hypothesis:

Large individual differences may exist in the rates at which smokers metabolize nicotine and also in the rates at which nonsmokers metabolize nicotine. We plan to investigate rates of nicotine metabolism in smokers and nonsmokers to determine whether such large individual variations exist in each group. If they do exist, the heavy smoker who is a slow metabolizer of nicotine might be exposed to higher and more prolonged blood and tissue levels of the drug than the rapid nicotine metabolizer. This possible difference in capacity to metabolize nicotine might render the slow nicotine metabolizer more liable to the pharmacological effects of nicotine. To investigate pharmacokinetic differences among individuals in nicotine metabolism, a radioimmunoassay for nicotine is being developed.

9. Details of experimental design and procedures (append extra pages as necessary)

After a brief unsatisfactory experience with 6-OH nicotine (which we found unsuitable to conjugate with albumin), we decided to synthesize another nicotine derivative that could be directly conjugated to albumin. The 6-amino-nicotine was synthesized according to the method of Chichibabin (Chem. Abstracts 19:69M, 1925) with several modifications in the scheme of purification:



The progress of the reaction was monitored with thin layer chromatography (TLC), using Methylene Chloride : Methanol, 4:1 as the solvent system and both silica gel and aluminum oxide as coatings for the plates. The spots were visualized either with short wave U.V. light or with Iodine vapor. Purification by column chromatography was attempted as separation on the plates was good. However, there was little correlation between separation on TLC and on column chromatography, probably because decomposition took place on the column even though the columns were protected from direct light.

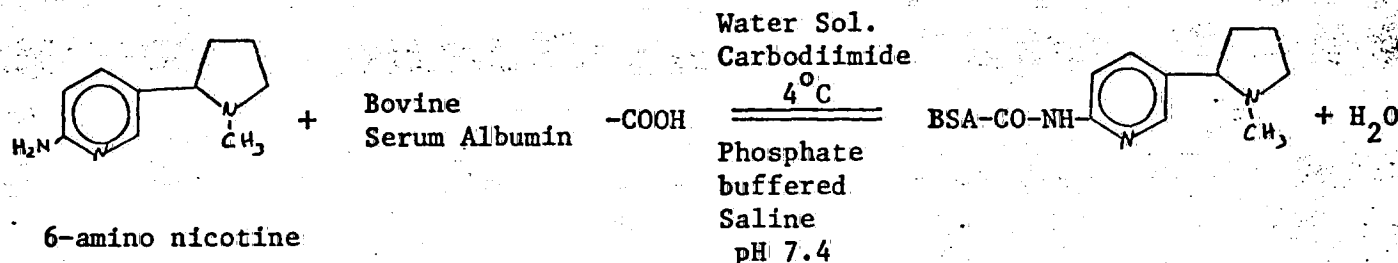
Rapid column chromatography was substituted and proved satisfactory, provided the extracted reaction mixture was purified partially by Kugelrohr distillation prior to chromatography. Two isomers of the amino nicotine, shown in the above equation, were found as predicted by Chichibabin. However, since his original paper was published in 1925 we decided to verify the assignment of structure of the two compounds by both infrared and NMR spectroscopy. The purity of the compounds was proved by these techniques and further assured by elemental analysis: the actual percentages agreed exactly with theoretically expected values. The 2-amino isomer was easily isolated, but the 6-amino isomer was considerably more difficult to isolate in crystalline form due to lack of the intra molecular hydrogen bonding present in the 2-amino compound. In addition, the 6-amino nicotine isomer is especially sensitive to light or air oxidation and is hygroscopic. It was partially purified by Kugelrohr distillation followed by rapid column chromatography, recrystallization from isopropyl ether and finally sublimation.

Several attempts were made to conjugate 6-amino-nicotine directly to bovine serum albumin (BSA) by a carbodiimide condensation procedure similar to that used by Spector and Parker (Science 168:1347, 1970), for their conjugation of morphine to BSA. The conjugate was examined by U.V. spectroscopy following ultrafiltration. The mole ratio of nicotine to BSA was not determined.

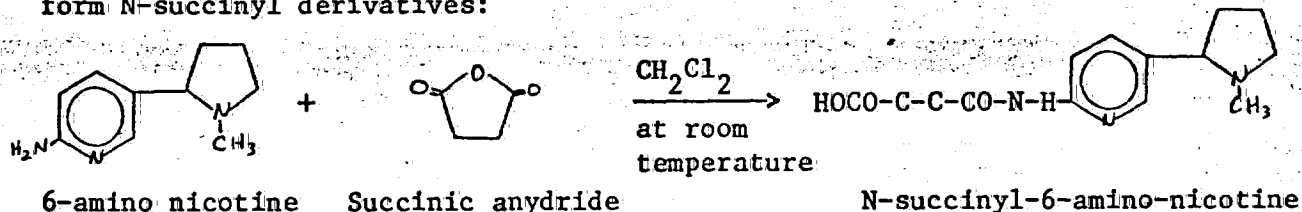
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9. Details of experimental design and procedures (cont'd.)

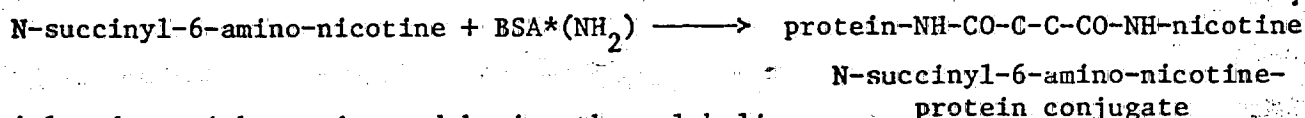
All immunizations were made in rabbits in suspensions of complete Freund's Adjuvant. Booster injections in multiple sites followed at the third week after injection. Samples were withdrawn weekly beginning two weeks after the booster injection. The antiserum was screened for nicotine sensitive antibodies with the Ouchterlony immunoprecipitation technique. The results were negative in all cases. Failure was thought to be due to too close approximation of the nicotine molecule to the albumin to permit recognition of the nicotine hapten by the antibody forming mechanism:



A new approach was therefore selected to place the nicotine hapten further away from the albumin molecule and thereby facilitate its recognition by the antibody forming process. Succinic anhydride reacts with amino groups to form N-succinyl derivatives:



This is a relatively long chain with no molecular substitutions so that it should have the smallest antigenic effects of its own. This compound has been synthesized and conjugation achieved:



*also done with porcine and bovine thyroglobulin

The conjugate was injected into rabbits one week ago with the expectation that an antibody to nicotine will be formed. Crossreactivity of such an antibody to cotinine will be tested. Although crossreactivity is expected, the extent to which cotinine and nicotine coexist in human blood for short periods after cigarette or cigar smoking can be determined independently by TLC. At very short periods after inhalation of smoke, cotinine will probably not be present in significant concentrations since more time would be required for its production by metabolism of nicotine. Thus, use of the radioimmunoassay would hopefully detect nicotine without its metabolites over short time periods after smoke inhalation. Fortunately for this project, nicotine disappears very rapidly from blood so a pharmacokinetic study of nicotine seems feasible to conduct by means of a radioimmunoassay for nicotine, even though crossreactivity of the antibody with cotinine may occur.

Following initial characterization, rabbit antinicotine sera will be further examined for titre, sensitivity and affinity, according to the method of Berson and Yalow (Clin. Chim. Acta 22:51, 1968). A standard curve will then be prepared from each antiserum; percent of radioactive nicotine bound to antibody is plotted against varying known concentrations of nicotine added in the presence of a constant predetermined amount of labelled nicotine. Cross reactance of such

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9. Details of experimental design and procedures (cont'd.)

chemically related compounds as cotinine, nicotinamide, and nicotinic acid will be determined for each antiserum by preparing standard curves for each compound.

The development of the nicotine radioimmunoassay at this stage should be relatively straightforward and will proceed along the following lines:

Selection of appropriate volumes of antisera, sample sera and tritiated nicotine will be determined. The necessity of maintaining sufficient radioactivity levels for efficient counting over the physiological nicotine concentration range will influence choice of these volumes.

Optimum times and conditions for incubation of antisera, sample sera, and tritiated nicotine vary with individual antisera and must be worked out empirically.

Separation of the bound and free phases is accomplished by adsorption of the free phase to either silica gel, dextran coated charcoal or similar adsorbent.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Laboratories of the Department of Pharmacology comprise 9000 square feet, including a cold room. Major equipment available therein includes:

Gilford Model 2400-S with recorder

Beckman Model DBG

Beckman Model DU2

Sorvall Model RC2B Centrifuge

Two Beckman L265B Centrifuges

International Model CL Centrifuge

Beckman DPM-100 Liquid Scintillation Counter

Aminco-Bowman Spectrofluorometer

Beckman Zeromatic SS-3 pH Meter

Dubnoff-Metabolic Shaking Incubator

Several Mettler P1200 Balances

Buchiflashevaporator

Brinkman Thin-Layer Equipment

High Temperature Oven

High-Speed Liquid Chromatograph, duPont 830

Glowall 320 Gas Chromatograph with 3 different detector systems

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

Appended

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Appended

14. First year budget:

A. Salaries (give names or state "to be recruited")
Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

Elliot S. Vesell

10

G. Thomas Passananti

100

21,000

Technical

Caroll Haines

50

3,000

Sub-Total for A

24,000

B. Consumable supplies (by major categories)

Reagents (including \$500 for tritiated nicotine)
Rabbits for antibody
Glassware

2,000

600

600

Sub-Total for B

3,200

C. Other expenses (itemize)

Human volunteers (30 @ \$100)

3,000

Sub-Total for C

3,000

Running Total of A + B + C

30,200

D. Permanent equipment (itemize)

Sub-Total for D

E

4,530

E. Indirect costs (15% of A+B+C)

Total request

34,730

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2						
Year 3						

1003539000

5.

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

None

CURRENTLY ACTIVE

Title of Project

Source
(give grant numbers)

Amount

Inclusive
Dates

PENDING OR PLANNED

Title of Project

Source
(give grant numbers)

Amount

Inclusive
Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

☒ Pennsylvania State University

Mailing address for checks

R. A. Patterson, Senior Vice President
for Finance and Operations
The Pennsylvania State University

University Park, Penna. 16802

Principal investigator

Typed Name Elliot S. VesellSignature Elliot S. Vesell Date 1/23/73Telephone 717 534-8285

Area Code

Number

Extension

Responsible officer of institution

Typed Name Thurman GrossnickleTitle Assistant Provost for Grants and Contracts
Hershey Medical CenterSignature Thurman Grossnickle Date 1/29/73Telephone 717-534-8495

Area Code

Number

Extension

1003539001

#680 AMPLATZ

1003539002

MISCELLANEOUS

1003539003

#880 AMPLATZ

1003539004

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

September 28, 1972

Grant Application No. 880

5
Denied

To: The committee comprising Drs. Bing, Cattell, and Jacobson
Subject: Kurt Amplatz, M.D., University of Minnesota, Minneapolis
New application No. 880
"The Acute Effects of Smoking on the Coronary Artery
System in Human Subjects"

History

This proposal was Case No. 116 and full application was encouraged by the Planning Committee.

Application No. 880 requests \$51,449, plus two additional years.

Document Submitted (attached)

Application dated July 17, 1972 (9 pages)

Comment

The first application received included studies of myocardial perfusion using Xenon 133, and requested some \$100,000 for purchase of the necessary equipment. On advice of CTR staff Amplatz elected to limit initial studies to angiography, and to seek funds from other sources for the Xenon 133 studies for which he is not at present equipped.

F.W.N.
F.W.N.

FWN:wg
Encl.

1003539005

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

COMMITTEE:

Dr. Bing
Dr. Cattell
Dr. Jacobson

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

Application For Research Grant

Date: July 17, 1972

1. Name of Investigator(s): (include Title and Degrees)

Kurt Amplatz, M. D., Professor of Radiology

2. Institution

Richard Morre, Ph. D., D. Sc., Associate Professor of Radiology

Address:

University of Minnesota Hospitals
Department of Radiology
Minneapolis, Minnesota 55455

3. Short Title of Project:

The Acute Effects of Smoking on the Coronary Artery System in Human Subjects.

4. Proposed Starting Date: As soon as the project is funded

5. Anticipated Duration of this Specific Study: Three years

Brief Description of Objectives or Specific Aims:

There is considerable controversy over the effects of smoking on the cardiovascular system in humans. While there has been much basic research in dogs, human studies have been primarily related to epidemiological studies and post-mortem examinations (1,2). An association between smoking and increased cardiovascular disease has been demonstrated. However, casual relationships have not been defined. Experiments in human subjects relating smoking to acute effects have primarily been electrocardiographic studies and apex cardiograms (3). These studies have indicated a relative myocardial ischemia with increased end diastolic filling pressure of the left ventricle and increased incidence of anginal attacks. The hypothesis is that there is a relative myocardial ischemia secondary to the increased work load of the heart caused by the effects of nicotine. Results from animal experiments confirm the hypothesis. It is our purpose to confirm or refute this hypothesis by performing coronary angiography, left ventriculography, and diastolic pressures, and Xenon 133 myocardial perfusion studies on patients before and after smoking. Only human subjects who are smokers will be used in this study because the effect of nicotine in these subjects might be different from the effect on animals which are obviously nonsmokers.

7. Give a Brief Statement of your Working Hypothesis: Nicotine has been shown in dogs to produce increased coronary blood flow, increased myocardial work load, and increased myocardial oxygen consumption (4,5). It has been hypothesized that this holds true for the human smoker also, but this hypothesis has never been substantiated. Using the tools of coronary angiography, left ventriculography, and diastolic pressures, and Xenon 133 perfusion studies, we hope to be able to prove or to disprove this hypothesis.

1003539006

2.
8. Details of Experimental Design and Procedures: (Attach Separate Pages)

Patients with suspected coronary artery disease who are smokers will undergo selective coronary arteriography, and the coronary arteries will be visualized radiographically in at least three projections. The exact diameter of the coronary arteries and areas of narrowing will be measured on the films and expressed in millimeters and percentage narrowing. The patient will be allowed to smoke a cigarette of his choice, a repeat coronary arteriogram will be performed in the identical lateral projection, and the diameter of the coronary arteries and areas of stenoses will again be measured and compared with the presmoking study. By this comparison, we hope to clarify the question whether smoking is a dilator or vasoconstrictor of the coronary arteries. In addition, the transit time of the contrast medium through the vascular bed will be compared. Any change of transit time will strongly suggest a change in capillary resistance due either to vasodilatation or to vasoconstriction.

In another group of patients, end diastolic pressures of the left ventricle will be recorded before and after smoking a cigarette in order to detect any changes in left ventricular function.

The most significant physiologic evaluation will be carried out in another group of patients for whom myocardial tissue blood flow will be measured using the Xenon 133 washout technique. Radioactive Xenon dissolved in saline will be injected selectively into the left coronary artery before and after smoking. Distribution of the isotope and its regional (see attached sheet)

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

Basic Equipment: 1200 MA three-phase generator with rapid roll film see-through changer and 35 mm. cine recording. Physiologic recording equipment for pressures and dye dilution studies. Power injector, defibrillator, automatic processors, etc. Other catheterization laboratories are equipped with biplane film changers.

10. Additional Requirements:

1. A gamma camera with dedicated computer will be mandatory to perform the most important part of this study.

11. Biographical sketches of all principal and professional personnel (append)

12. List of publications: (Five most recent as pertinent) (append)

1003539007

3.

13. Budget (1st year)

A. Salaries (Personnel by names)

% time

Amount

Professional

Kurt Amplatz, M. D., Professor of Radiology
 Research Fellow, to be appointed
 Richard Moore, Ph. D., Program Analyst

20%
 100%
 50%

Technical

X-Ray Technician
 Isotope Technician
 Secretary

50%
 50%
 20%

Sub-Total

B. Consumable Supplies (list by categories)

Drugs, contrast media, isotopes, computer
 paper, catheters, guidewires, x-ray film,
 sterile supplies, etc.

Sub-Total

\$ 4,000

C. Other Expenses (itemize)

Travel

1,000

Sub-Total

\$ 1,000

D. Permanent Equipment (itemize)

E. Overhead (15% of A+B+C)

\$ 6,711

Total

\$ 51,449

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2		4,000	1,000		7,065	54,165
Year 3	REDACTED	4,000	1,000		7,440	57,040

It is understood that the applicant and institutional officers
 in applying for a grant have read and found acceptable
 the Council's "Statement of Policy Containing Conditions
 and Terms Under Which Project Grants Are Made."

Signature

Director of Project

Signature

Business Officer of the Institution

Telephone

Telephone

1003539008

Continuation of #8

washout will be recorded by a gamma camera, and washout curves will be analyzed by a dedicated computer. Data will be collected, tabulated, and submitted for statistical analysis. From the results, it is hoped to clarify the controversial question of the anatomic and hemodynamic effects of smoking on the human heart. There should not be any significant added hazard. The patient will sign a consent form. The project is presently being considered by the human experimentation committee. The study will be performed in the laboratories of the University of Minnesota Hospitals.

1003539009

Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current

Title of Project	Source	Amount	Duration
Coronary Revascularization Using an Anger Camera	National Institute of Health	\$ 165,103.00	3 years
Clinical Training in Cardiovascular Radiology	National Institute of Health	\$ 524,938.00	4 years

Pending

Coronary Revascularization Using an Anger Camera (request for supplemental films)

National Institute of Health	\$ 67,310.00
------------------------------	--------------

1003539010

SECTION II - PRIVILEGED COMMUNICATION			
BIOGRAPHICAL SKETCH			
(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)			
NAME Amplatz, Kurt	TITLE Professor of Radiology	BIRTHDATE (Mo., Day, Yr.) R	
PLACE OF BIRTH (City, State, Country) R	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date) R	SEX <input checked="" type="checkbox"/> Male <input type="checkbox"/> Female	
EDUCATION (Begin with baccalaureate training and include postdoctoral)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Gymnasium, Innsbruck, Austria University of Innsbruck	M. D.		Medicine
HONORS Summa cum laude, University of Innsbruck, Austria			
MAJOR RESEARCH INTEREST Angiocardiography		ROLE IN PROPOSED PROJECT Project Director	
RESEARCH SUPPORT (See instructions) "Clinical Training in Cardiovascular Radiology", PHS 2 T01 HL 5853-04A1, July 1, 1972 - June 30, 1976. \$124,382.00 for four year period. "Coronary Revascularization Using an Anger Camera", PHS 1 R01 HE 13998-01, June 1, 1971 - August 31, 1972. \$71,185 for first 15 months. Recommended support for next two years, \$53,679 and \$40,239.			
RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List all or most representative publications. Do not exceed 3 pages for each individual.) Professor, University of Minnesota, 1970 Associate Professor, University of Minnesota, 1963 Assistant Professor, University of Minnesota, 1961 Instructor, University of Minnesota, 1957 Received medical training in Europe at the Universities of Friburg, Switzerland; Zurich, Switzerland; Paris, France; and Innsbruck, Austria Residence training in diagnostic radiology, therapy, and nuclear medicine at Wayne State University, Detroit, Michigan.			

AHS-J08
Rev. 3-70

U. S. GOVERNMENT PRINTING OFFICE : 1971 O - 411-174

10035339011

SECTION II - PRIVILEGED COMMUNICATION

BIOGRAPHICAL SKETCH

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME Richard Moore	TITLE Associate Professor	BIRTHDATE (Mo., D.) R
PLACE OF BIRTH (City, State, Country) Los Angeles, California, USA	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date) U.S. Citizen	SEX <input checked="" type="checkbox"/> Male <input type="checkbox"/> Female
EDUCATION (Begin with baccalaureate training and include postdoctoral)		
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED
University of Missouri, Columbia, Mo.	B.S.	
University of Rochester, New York	Ph.D.	
George Washington University, Wash. D.C.	D.Sc.	
		SCIENTIFIC FIELD
		Electrical Engineering
		Biophysics
		Biomedical Engineering

HONORS Member:

REDACTED

REDACTED

MAJOR RESEARCH INTEREST Analysis of Physiological Data	ROLE IN PROPOSED PROJECT Biostatistician and Programmer-Analyst
---	--

Special Qualifications:

Dr. Moore is an Associate Professor of Biometry and a member of the American Statistical Society. He is familiar with the application of statistical methods to experimental data. He is certified by the Processing Management Association. He has fifteen years' experience with computers, and he is an Associate Professor of Health Computer Sciences. He has over sixty publications in these fields.

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List all or most representative publications. Do not exceed 2 pages for each individual.)

PROFESSIONAL EXPERIENCE:

- 1969 - Present:
University of Minnesota, Department of Laboratory Medicine.
Associate Professor (Joint Appointments in Biometry, Radiology, and Biophysics).
- 1960 - 1969:
American National Red Cross, Washington, D.C. and Bethesda, Maryland
Chief, Biophysics Section, Blood Research Laboratory.
- 1968 - 1969:
George Washington University, Washington D.C.
Visiting Professor (Radiation Biology), Department of Radiology.
- 1965 - 1968:
George Washington University, Washington, D.C.
Visiting Assistant Professor, Department of Physiology.
- 1957 - 1960:
National Institutes of Arthritis & Metabolic Diseases
Scientist
- 1955 - 1957:
Public Health Service
Commissioned Officer Radiological Health Program.

1003539012

12. List of publications: (Five most recent as pertinent).

Kurt Amplatz, M. D.

1. White, R. I., Jr.; Frech, R. S.; Castaneda, A.; and Amplatz, K.: The Nature and Significance of Anomalous Coronary Arteries in Tetralogy of Fallot. Am. J. Roentgenol. Radium Ther. Nucl. Med. 114: 2, pp 350-354, February, 1972.
2. White, R. I., Jr.; Frech, R. S.; and Amplatz, K.: An Improved Technique for Right Coronary Artery Catheterization. Am. J. Roentgenol. Radium Ther. Nucl. Med. 113: 3, pp 562-566, November, 1971.
3. Chapter "The Value of Vectorcardiography, Electrocardiography and Exercise Electrocardiography in the Diagnosis of Coronary Artery Disease. Correlation with Coronary Arteriography." in VECTORCARDIOGRAPHY 2. Naip Tuma, M. D.; Gerald B. Lee, M. D.; and Kurt Amplatz, M. D. Proceedings of the XI International Symposium on Vectorcardiography. North-Holland Publishing Company. Editor I. Hoffman, Co-editors R. I. Hamby and E. Glassman, 1971.
4. Snyder, C.; Cramer, R.; and Amplatz, K.: Isolation of Sodium as a Cause of Ventricular Fibrillation. Invest. Radiol. 6:245-248, July-August, 1971.
5. Loken, M. K.; White, R. I., Jr.; Ponto, R. A.; Frech, R. S.; and Amplatz, K.: Intravenous Radioisotope Angiography with Computer Processing of Data. (abstract). J. Nucl. Med. 12: 448, June, 1971.

Richard Moore, Ph. D.

1. Moore, R.; Ledley, R. S.; and Sing, H. C.: Application of Automatic Processing Methods to the Radiologic Image. The radiologic Clinics of North America, 7: 473-483, December, 1969.
2. Moore, R. and Wingert, R. A.: Calibration of Laboratory Instruments by Computer. Medical Electronics and Data, 1: 76-82, April, 1970.
3. Moore, R. and Ledley, R. S.: Evaluation of the Significance of Coherent Scattering of an X-Ray Beam to Darkening of Radiographic Film. (In) Proceedings of Bone Measurement Conference. (Ed.) J. R. Cameron, Atomic Energy Commission, CONF-700515, pp. 205-233, 1970.
4. Moore, R.; Ledley, R. S.; and Sing, H. C.: Application of Automatic Processing Methods to Radiologic Images. Yearbook of Radiology, 10-11, 1971.
5. Moore, R.: Computer Calculation of Ventricular Volume From Roentgenograms. Medical Electronics and Data, 2: 56-61, 1971.

1003539013

REFERENCES

1. Auerbach, O., et al.: Thickness of Walls of Myocardial Arterioles in Relation to Smoking and Age. Arch. Environ. Health 22:20-27, January, 1971.
2. Seltzer, C. C.: The Effect of Cigarette Smoking on Coronary Artery Disease. Arch. Environ. Health 20: 418, March, 1970.
3. Aronow, W. S.: The Effect of Smoking Cigarettes on the Apexcardiogram in Coronary Heart Disease. Chest 59: 365-368, April, 1971.
4. Ross, G. and Blesa, M. I.: The Effect of Nicotine on the Coronary Circulation of Dogs. Amer. Heart J. 79: 96-102, January, 1970.
5. Leb, G., et al.: The Effect of Nicotine on Effective and Total Coronary Blood Flow in the Anesthetized Closed-Chest Dog. J. Pharmacol. Exp. Ther. 173: 138, May, 1970.

1003539014

#898 HELINSTEIA

1003539015

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

1

January 30, 1973

Grant Application No. 898

To: The committee comprising Drs. Andervont, Huebner, and Meier

Subject: Norman W. Heimstra, Ph.D., University of South Dakota,
Vermillion, South Dakota
New application No. 898
"Effects of Smoking Deprivation on Group Problem Solving
Processes"

History

CTR has supported related studies by Dr. Heimstra going back to 1964.

The pending request is for \$12,592, plus one additional year.

Documents Submitted (attached)

Application dated 1/22/73 with appendices.

Comment

Staff will attempt to obtain an outside evaluation of this proposal.


F.W.N.

FWN:wg
Encls.

1003539016

MEMORANDUM

5 February 1973

To: DR. F. W. NORDSIEK

From: H. MEIER

Subj: APPLICATION NO. 898 BY N. W. HEIMSTRA, "EFFECTS OF SMOKING
DEPRIVATION ON GROUP PROBLEM SOLVING"

Since I am not a behaviorist, I asked one of my colleagues in the psychology group here at the laboratory to comment on Heimstra's application. His comments to me are as follows: "Although the ultimate goal of the project is to analyze the effects of the prohibition of smoking on group decision-making processes in groups of smokers, certain seemingly critical points are overlooked.

a) In the real world, decision-making groups are seldom comprised, exclusively, of either smokers or non-smokers. The behavior of deprived smokers may not be the same as that of a group of deprived smokers plus non-smokers. It would seem, on an intuitive basis, that a knowledge of the effects of deprivation on individual smokers would be a good predictor of behavior of a group of deprived individuals.

On page 2D, the author states that the effect of smokers in a group on the behavior of non-smokers is an interesting problem that isn't covered. I think it is more than an interesting problem; it is a problem germane to the overall goals of the proposal. I would say that the design of the experiment would be more meaningful if the interactions between non-smokers, smokers, and deprived smokers were also included in the investigation, rather than restricting all of the experimental groups to members all of one type or another.

b) Each experimental condition (non-smokers, smokers, and deprived smokers) includes 15 triads. This seems a relatively small sample in view of the tremendous opportunity for individual differences, independent of smoking habits. No controls exist for general social attitudes, decision-making capabilities, personality conflicts, etc., which the individual carries into the experimental situation. An individual's smoking behavior may also be the effect of, rather than the cause of various personality characteristics, and his behavior, rather than the behavior of a "comparable control group" may necessarily have to be analyzed prior to altering the conditions of his smoking behavior.

c) In regard to screening the individuals prior to assigning them to groups: the assumption implicit in the design is that a person smoking a pack of cigarettes per day will react to deprivation in the same manner, and to the same extent as an individual smoking three packs of cigarettes per day. I find this difficult to believe.

1003539017

In summary, since this is a study of social behavior, and concentrates on the subtle interactions of social phenomena, personality variables, and deprivation of a behavior which has both social and physiological (not to mention personal psychological) ramifications, I think the pay-off is rather small considering the expenditure of time, energy and money. The study will, or may, provide some information on the interactive effect of individuals who are being deprived of the physiological aspects of smoking, e.g. withdrawal from temporary, intermittent hypoxia, or from nicotine, but we already have information on the effects of these variables on individual behavior. Therefore, I think that the applicant should take more steps to investigate the more subtle and elaborate interactions and consequences of mixing smokers, non-smokers, and deprived smokers in decision-making situations."

HM:tg

cc: Drs. Andervont and Huebner

(R (by PTR)
WAG
RCH
JCS

1003539018

Comm.

Dr. Andervont
Dr. Huebner
Dr. Meier

MISCELLANEOUS

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8985

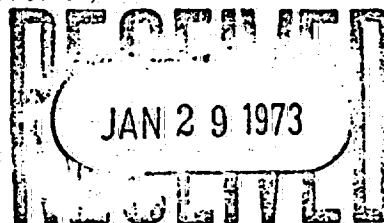
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#708-1/1/70-6/30/71
#641-6/1/68-6/1/69
#527-6/1/66-12/1/67
CF421-6/1/64-6/1/65

Application for Research Grant
(Use extra pages as needed)

Date: 1/22/1973

1. Principal Investigator (give title and degrees):

Norman W. Heimstra, Ph.D.
Professor of Psychology and Director,
Human Factors Laboratory



2. Institution & address:

University of South Dakota
Vermillion, South Dakota 57069

3. Department(s) where research will be done or collaboration provided:

Department of Psychology

4. Short title of study:

Effects of Smoking Deprivation on Group Problem Solving Processes

5. Proposed starting date: September 1, 1973

6. Estimated time to complete: Two years

7. Brief description of specific research aims:

Increasingly, smokers are encountering situations where they are not allowed to smoke because of newly imposed restrictions. These situations range all the way from those encountered in public transportation systems to legislative committee meetings (see attached exhibit). While these restrictions are imposed because someone in authority views smoking as annoying or as a health hazard, little thought is given to the possible detrimental effects that smoking deprivation may have in terms of performance of the deprived smokers in, for example, committee meetings where problems are solved and decisions are made. The specific aims of this investigation are:

- 1) to study group problem solving efficiency under conditions where smokers are deprived, or are allowed to smoke to determine if smoking deprivation interferes with group processes involved, and
- 2) to systematically observe and record the group interactions to determine if differences exist between groups of smokers who are deprived or allowed to smoke. Of particular interest will be frequency of occurrence of behaviors categorized as aggressive, antisocial, etc.

1003539019

Previous research has shown that performance on various types of tasks and affective states, such as mood, are negatively affected when smokers are not allowed to smoke. These studies have involved individuals rather than groups. The working hypothesis is that because of the stress and affective states developed by forced smoking deprivation, group performance where members are deprived smokers will be less effective than performance in groups where smokers are allowed to smoke and in groups involving non-smokers. It is also hypothesized that the type of social interaction demonstrated by the deprived smoker groups will differ qualitatively from that in the other groups and that more "anti social" type behavior will be shown.

9. Details of experimental design and procedures (append extra pages as necessary)

Introduction

In earlier investigations by the applicant dealing with the effects of smoking on behavior, the experimental design has called for conditions where smokers were deprived. In several studies, marked differences in performance were noted between conditions where smokers were allowed to smoke and where smoking was not permitted. For example, in a study concerned with the effects of smoking on sustained performance in a driving simulator, deprived smokers performed significantly poorer than subjects who were allowed to smoke (Heimstra, Bancroft, & DeKock, 1967). Similar trends were found in a study designed to determine the relationship between smoking, psychomotor performance, and stress (Bancroft, Heimstra, & Warner, 1967). In studies where the mood of smokers and deprived smokers has been measured before and after exposure to various tasks, the mood of the deprived smokers has shown a great deal more instability than that of the smokers (Heimstra, 1973). In all of these investigations, it was obvious that the deprived smokers were under considerable stress and that their behavior and attitude toward the experimenter differed considerably from that of the smoker and non-smokers.

While the design of these experiments was such that subjects were tested individually, an interesting question is raised concerning the effects that smoking deprivation might have on group performance, i.e., situations where several individuals are required to contribute to the solution of a problem or make a decision. There has been an increasing trend toward banning smoking in staff meetings, committee meetings, etc. At the time this proposal is being written, the South Dakota Legislature is in the process of banning smoking in legislative committee meetings (see attached exhibit). While the justification for banning smoking is presented in terms of annoyance of non-smokers and possible health hazards involved for non-smokers, very little concern has been expressed regarding the possible detrimental effect smoking may have on the functioning of the various kinds of groups that are involved. The proposed investigation is designed to determine if, in fact, smoking deprivation has demonstrable effects in terms of group performance on specific tasks and also in terms of the types of social interactions demonstrated by members of the groups.

1003539020

Methods

The basic design of the proposed study is similar to that involved in previous research by the applicant dealing with smoking and behavior. Three conditions are utilized - one involving non-smokers, another involving smokers who are not allowed to smoke (smoker deprived) and a third condition involving smokers who are allowed to smoke. However, where previous studies have been concerned with the performance of individual subjects, the proposed study will deal with the performance of groups of subjects under these conditions. Groups of three subjects each (triads) will be utilized. A number of groups will be studied under each of the conditions, i.e., groups of three non-smokers, smokers, and deprived smokers, and their performance on a problem solving task obtained and recordings of social interaction made.

Group Problem Solving Task

The variables affecting group problem solving has been subjected to a great deal of research, much of which has been summarized by Kelley and Thibaut (1969). In these studies a wide variety of tasks have been utilized ranging from psychomotor tasks to those which are aimed at the most complex intellectual processes such as concept formation, detecting relationships, analogical reasoning, etc. The specific problem solving task to be used in the proposed study will be determined after a comprehensive search of the literature is made and after some pilot work has been conducted. The task selected for use will have to meet several important criteria: (1) It will be such that input from all three members of the group is necessary in order for progress to be made in arriving at a solution. (2) There will be various "stages" of solution each of which must be arrived at before the next stage can be solved. This will allow for a series of measures which can be obtained during the duration of the session, i.e., how long it took to solve each stage of the task and how accurate the solution was. (3) The problem will be such that a minimum of three hours will be required for its solution and possibly up to five hours. Since, in the "real world," committees are often required to meet until a particular problem is solved and a decision arrived at, this will add realism to the laboratory setting involved in the proposed study. Payment of the subjects will be based on a solution to the problem, not for the amount of time utilized. (4) It is necessary that the task be quantifiable in terms of duration and accuracy. If possible, the task will be such that not only will the performance of the group be measured but also the performance of each member of the group. (5) Several versions, or alternate forms, of the task will be developed in order to reduce the problem of communication about the nature of the task among the subjects tested at different times.

As indicated, the specific details of the problem solving task will be determined based on an extensive literature review and on exploratory research.

Systematic Observation of Group Interactions

There is a considerable amount of research in the behavioral sciences where behavior of interest to the investigator is categorized, subjects are observed, and the frequency of behavior demonstrated in the various categories is recorded. The applicant has conducted a number of different studies using this method with subjects ranging from fish, rats, and monkeys to children as they crossed streets. (see attached vita).

1003539021

9. Continued

The purpose of analyzing the interactions of group members in the proposed study is to determine whether the interaction within groups who have been deprived of smoking differs in any way from the interactions shown within the groups in the other conditions. There have been several systems of behavior categories developed within the last few years to encode social interaction and these are reviewed by Weick (1968). The method that appears to be most applicable to the proposed study is the Interaction Process Scores (IPS) developed by Bales (1950) and refined by Borgatta (1963) which has been used in a wide variety of studies in recent years. The IPS categories are listed below. Observers trained on this system show high between-observer reliability.

- 01 Common social acknowledgments
- 02 Shows solidarity through raising the status of others
- 03 Shows tension release, laughs
- 04 Acknowledges, understands, recognizes
- 05 Shows agreement, concurrence, compliance
- 06 Gives a procedural suggestion
- 07 Suggests a solution
- 08 Gives opinion, evaluation, analysis, expresses feelings or wish
- 09 Self-analysis and self-questioning behavior
- 10 Reference to the external situation as redirected aggression
- 11 Gives orientation, information, passes communication
- 12 Draws attention, repeats, clarifies
- 13 Asks for opinion, evaluation, analysis, expression of feelings
- 14 Disagrees, maintains a contrary position
- 15 Shows tension, asks for help by virtue of personal inadequacy
- 16 Shows tension increase
- 17 Shows antagonism, hostility, is demanding
- 18 Ego defensiveness

With this system of categories, the observer(s) record each time a subject in a group engages in behavior defined by a particular category. In the proposed study, the groups will work in a room which has been equipped with a concealed video-tape system. A time sampling technique will be used where the group interactions will be taped for certain periods of time during each hour, e.g., five minutes of taping every 15 minutes. The exact schedule will be determined by pilot work. Thus, instead of actually observing the subjects and categorizing the behavior directly, observers will categorize from the video-tapes. This allows for playback of behavior which may be difficult to categorize and for a consensus to be arrived at by several observers. In addition to the categories listed, additional information such as number of cigarettes smoked, will also be obtained.

General Procedures

The exact procedures to be utilized will depend upon the specific problem solving task selected for use in the study. In many studies on group problem solving behavior, it is necessary to familiarize subjects with the problem, explain procedures, etc., before the sessions are actually started. It is probable that this will be the case in the proposed study.

1003539022

Subjects will be selected on the basis of a questionnaire that has been utilized in a number of previous studies in this laboratory. Non-smokers are defined as individuals who have not used tobacco in any form for at least one year prior to answering the questionnaire. A smoker is defined as one who regularly smokes twenty or more cigarettes per twelve-hour waking day; who uses this form of tobacco exclusively or primarily; who consistently inhales; and who has so smoked for a period of at least one year prior to answering the questionnaire. When completing the questionnaire, usually in a University class of some sort, the individuals are under the impression that it is a smoking behavior survey. They are not aware that their qualifications as a potential subject in an experiment is determined on the basis of their responses. Individuals who qualify are contacted and asked if they wish to participate in a research project for which they will be paid. Subjects will be male college students.

Fifteen groups, each group consisting of three members, will be studied under each of the three conditions. Thus, 15 groups of non-smokers will be utilized, 15 groups of smokers who are allowed to smoke during the task, and 15 groups of smokers who are not allowed to smoke during the task. During test sessions no food or drink will be permitted under any of the conditions although individuals will be allowed to use toilet facilities (which adjoin the test room) and obtain a drink of water if they so desire. All smoking materials will be removed from subjects in the smoker condition and smoker-deprived condition before entering the test room. However, subjects in the smoker condition will be furnished with a choice of cigarettes in the room. This precludes the possibility of some unidentified smoking material, e.g., marihuana, being taken into the test situation.

Including the training time and a minimum of three hours in the test session, each subject will be involved in the study for at least four hours and possibly up to six hours. Subjects will not be paid on an hourly basis but, rather, for completing the problem. A bonus will be involved for rapid and accurate completion. Thus, a group will receive \$18 (\$6 for each subject) if they have spent the full five hours allowed and have not completed the problem. However, if they complete the problem in four hours they will receive the \$18 plus an additional \$6 (a total of \$8 per subject) and if they finish the problem in three hours they will receive the \$18 plus \$12 as a bonus or a total of \$30 per subject. A system such as this will be necessary in order to insure a high level of motivation on the part of the subjects and a serious effort at solving the problem. It will also create an atmosphere which will put a considerable amount of pressure on the subjects and which may enhance the possible effects of smoking deprivation. The above system will be used in pilot work and some modifications may be necessary but a remuneration system along these lines will be necessary.

It is difficult, of course, to duplicate in a laboratory setting all of the variables that might be of importance in the "real world." For example, the situation for the smoker-deprived subjects in the experiment will be somewhat more strenuous than might be encountered in the "real world" situation where an individual in a group where smoking was banned would probably "sneak" out for an occasional cigarette. While it would be interesting to add other groups where "smoking breaks" were allowed, this would rapidly increase the scope of the study. Similarly, it would be desirable to utilize older subjects as well as students in order to include some groups where the members had been

1003539023

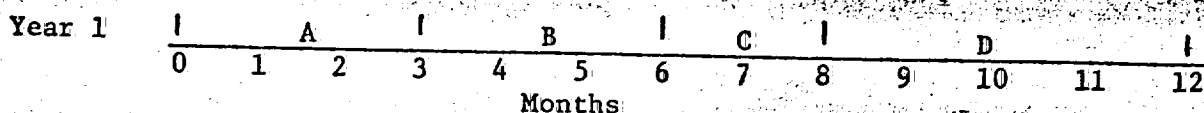
smoking for many years. Other interesting questions, such as the effects of smoking by some members of a group on the performance and behavior of non-smoking members, are also of interest. If results from the proposed study indicate that smoking deprivation does, indeed, have a significant effect on group behavior, future research might be conducted which includes these kinds of variables.

Data Analysis

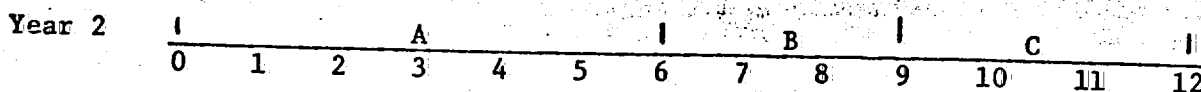
The data obtained from the measures of performance on the group problem solving task will be subjected to statistical analysis in order to determine whether differences existing between the performance of groups in the non-smoker, smoker deprived, and smoker condition, are significant. Measures of group performance, such as accuracy and speed, will be analyzed. However, an analysis will also be conducted on performance of the individuals within each group in order to determine whether patterns of individual performance differ between subjects in groups exposed to the different conditions.

Frequency of occurrence of the different categories of behavior obtained during the systematic observation of group interactions will be analyzed to determine if significant differences exist between the groups in the different conditions.

Time Frame



- A - Literature review and design of group problem solving test
- B - Pilot work and possible redesign of test
- C - Additional pilot work
- D - Subject testing



- A - Subject testing
- B - Data analysis
- C - Preparation of final report

References

- Bales, R. F. Interaction process analysis. Cambridge, Mass.: Addison-Wesley
- Bancroft, N.R., Heimstra, N.W. & Warner, H.D. Relationship between smoking, psychomotor performance, and stress. Final Report, Council for Tobacco Research, USA, 1967.
- Borgatta, E.F. A new systematic interaction observation system: behavior scores system. J. psychol. Stud., 1963, 14, 24-44.

1003539024

Heimstra, N.W. The effects of smoking on mood change. In W. L. Dunn (Ed.) Smoking behavior: Motives and incentives. Washington, D.C.: V. H. Winston & Sons, 1973.

Heimstra, N.W., Bancroft, N. R., & DeKock, A.R. Effects of smoking upon sustained performance in a simulated driving task. In H. B. Murphy (Ed.) The effects of smoking on the central nervous system. Ann. N.Y. Acad. Sci., 1967.

Kelley, H.H. & Thibaut, J.W. Group problem solving. In G. Lindzey and E. Aronson (Eds.) The handbook of social psychology (2nd ed., Vol.IV). Reading, Mass.: Addison-Wesley, 1969.

Weick, K. E. Systematic observational methods. In G. Lindzey and E. Aronson (Eds.) The handbook of social psychology (2nd Ed. Vol.II) Reading, Mass: Addison-Wesley, 1968.

1003539025

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The facilities of the Human Factors Laboratory are available for this study. A room with one-way mirrors and concealed video-tape equipment is available for testing groups. There is ready access to subjects and professional assistance in the form of advanced graduate students in psychology. Computer facilities available for data analysis.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

1003539026

4.
14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

Norman W. Heimstra

15

\$4200

Research Assistant (to be recruited-
Post MA level graduate student)

50

3600

Technical

Hourly assistance (observers, lab.
assistance, etc.) 400 hrs. at
\$3.00/hr.

1200

Sub-Total for A

\$9000

B. Consumable supplies (by major categories)

Video-tape supplies \$300
Test construction 500
Misc. 250

Sub-Total for B

\$1050

C. Other expenses (itemize)

Subjects for first year
(pilot work & actual testing
30 groups at \$30 each)

Sub-Total for C

\$900

Running Total of A + B + C

\$10,950

D. Permanent equipment (itemize)

None

Sub-Total for D

0

E. Indirect costs (15% of A+B+C)

E

\$1642

Total request

\$12,592

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	9500	600	1300	0	1710	\$13,110
Year 3						

Year 2

9500

600

1300

0

1710

\$13,110

Year 3

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE			
Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Accident Prevention Research	USPHS OH 00002-07	213,500	7/01/69 - 6/30/74
Effects of Smoking on Peripheral Movement Detection	US Army DADA17-73- C 3037	17,000	11/01/72 - 10/30/73
Noise and Human Performance	USPHS OH 00365-02	28,466	10/01/71 - 9/30/73

PENDING OR PLANNED			
Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Noise and Human Perf. (Renewal request)	OH 00365-03	30,041	10/01/73 - 09/30/75
(Risk-taking and Hazard Perception in Farm Accidents (proposal submitted)	USPHS	47,461	09/01/73 - 08/30/75

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

University of South Dakota

Mailing address for checks

Business Manager, Univ. of S.D.

Vermillion, South Dakota 57069

Principal investigator

Typed Name Norman W. Heimstra, Ph.D.

Signature Norman Heimstra Date 1/22/73

Telephone 605-677-5295

Area Code

Number

Extension

Responsible officer of institution

Typed Name Richard L. Bowen, Ph.D.

Title President

Signature Richard L. Bowen Date 1-24-73

Telephone 605-677-5641

Area Code

Number

Extension

1003539028

EXHIBIT

Joint Body Approves Rule To Ban Smoking At Meets

By TERRY DE VINE
Associated Press Writer

PIERRE (AP) — There may be a number of bills smoked-out of committee during the 1973 legislative session, but there aren't going to be any legislators smoked-out of committee, literally, if a joint rule approved Thursday gets through both Houses.

The joint rules committee approved by a 5-1 vote a rule that would ban all smoking during House and Senate committee meetings.

The motion to approve the no smoking rule was made by Sen. Oscar Austad, R-Sioux Falls, who said, "I think it's time the legislature started protecting the rights of nonsmokers on committees."

The Sioux Falls lawmaker said he would like to see the no smoking ban extended to the floors of both chambers and the House and Senate galleries, but settled for a more limited rule.

"I think the members of each committee have the right to breathe pure air," said Austad. "In light of recent smoking studies, I feel smokers are infringing on the rights of nonsmokers."

The only member of the joint rules committee who didn't side with Austad was Sen. Charles Donnelly, D-Rapid City.

"Eight months ago today I gave up a three-pack-a-day habit," said Donnelly, "but I feel we are infringing on the rights of smokers if we pass this rule."

Rep. John Bibby, R-Brookings, who voted with Austad even though he himself is a smoker, said, "committee rooms are one thing, but ban-

ning smoking in the chamber is another thing." The Brookings Republican said he felt prohibiting smoking on the floor of the Houses might affect the work of legislators because the smoker might be ducking out for a cigarette occasionally during floor action or be unduly nervous or uncomfortable because they couldn't smoke.

The rule, which must be approved by both houses, is not expected to come up for debate until next week and opposition is certain.

1003539029

VITA

Norman W. Heimstra

I. Biographical Data

Born: October 14, 1930. Mitchell, South Dakota
Married - Three children
Present Address: Department of Psychology
University of South Dakota
Vermillion, South Dakota

Present Position: Professor
Director, Human Factors Laboratory

II. Military History

Five years U.S. Navy--1948-1953

III. Academic History

B.A. Univ. South Dakota, 1955
M.A. Univ. South Dakota, 1956
Ph.D. Univ. of Rochester, 1960

IV. Related Experience

1. Research Assistant - Univ. South Dakota Primate Laboratory (1955-1956).
2. Teaching Assistant - Univ. of Rochester, 1956-1957.
3. Research Associate - HumRRO (Geo. Wash. Univ.) 1957-1959.
4. U.S.P.H.S. Predoctoral Fellow - Univ. of Rochester, 1959-1960.
5. Research Scientist - HumRRO 1960-1961.
6. U.S.P.H.S. Postdoctoral Fellow, Univ. of South Dakota, 1961-1963.
7. Assistant Prof., Univ. of South Dakota, 1963-1964.
8. Associate Prof., Univ. of South Dakota, 1964-1967.
9. Professor, Univ. of South Dakota, 1967-

1003539030

V. Membership in Organizations, Consultantships, Advisory Panels

APA, Human Factors Society,
American Association for Automotive Medicine
Sigma Xi (President, South Dakota Chapter, 1965-1966)
Highway Research Board Liaison Representative--University
of South Dakota
Member of Special Committee 11--Driving Simulation,
Highway Research Board, National Academy of Science
Consultant--Department of Army (Design and Measurement
of Performance)
Advisory Panel--American Institute for Research
Editorial Board--Human Factors

VI. Academic Experience

Courses Taught--Introductory Psychology
Physiological Psychology (undergraduate)
Seminar in Physiological Psych. (graduate)
Seminar in Psychopharmacology (graduate)
Industrial Psychology (undergraduate)
Personnel Selection & Training (graduate)
Human Factors Psychology (graduate)
Seminar in Accident Prevention Research
(graduate)

Ph.D. Dissertations Directed

Arthur McDonald (1966) Modification of agonistic
behavior in fish

Truman M. Mast (1966) Influence of motivational
variables on prerest and postrest performance
in rotary pursuit tracking

Norris Bancroft (1968) Relationship between smoking
and psychophysiological response to stress

Mark Hofmann (1968) A comparison of visual, auditory
and electrocutaneous displays in a compensatory
tracking task

Arlan DeKock (1968) Relationship between decision
making under conditions of risk and selected
psychological tests

Vernon Ellingstad (1969) A multivariate evaluation
of selected driver performance measures

David Damkot (1969) A comparison of auditory,
visual, and electrocutaneous displays in a
vigilance task

1003539031

Gary Martin (1970) Hazard perception by children

Richard Lucas (1970) Development and evaluation
of a part task film simulation technique for
training drivers on a critical passing skill

Thomas R. Schori (1970) A comparison of visual,
auditory, and cutaneous tracking displays
when divided attention is required to a
cross-adaptive loading task

Harold D. Warner (1970) Effects of intermittent
noise on visual search tasks of varying
complexity

Kent Kimball (1971) Combined task performance:
A study of the effect of coded signal processing
on a compensatory tracking task

John Fechter (1972) Effects of noise on skill acquisition

M.A. Theses Directed -- 25

1003539032

VII. Publications - Open Press and Technical Reports

1954

(With J. Cho) Perception of absolute size in a distorted room. Proc. S. Dak. Acad. Sci., 1954, 33, 44-47.

1955

(With R. T. Davis and M. Grodsky) Exploratory behavior in rats. Proc. S. Dak. Acad. Sci., 1955, 34, 93-103.

1956

(With H. Odio) Learning with food and non-food reward by rhesus monkeys. Proc. S. Dak. Acad. Sci., 1956, 35, 211-220.

1957

(With R. T. Davis and J. P. Steele) Effects of various food deprivation schedules on the discrimination learning performance of monkeys irradiated with X-Ray irradiation. J. Psychol., 1957, 44, 271-281.

1960

(With S. J. Goffard, R. S. Beecroft, and J. W. Openshaw) Basic electronics for minimally qualified men: An experimental evaluation of a method of presentation. HumRRO Tech. Report #61, 1960.

(With G. Newton) Effects of early experience on the response to whole body x-irradiation. Canad. J. Psychol., 1960, 14, 111-119.

1961

(With G. Newton) Effects of prior food competition on the rat's killing response to the white mouse. Behaviour, 1961, 14, 95-102.

Effects of chlorpromazine on dominance and aggressive behavior in the rat. Behaviour, 1961, 18, 313-321.

1962

Social influence on the response to drugs: I. Amphetamine sulfate. J. Psychol., 1962, 53, 233-244.

Effects of amphetamine sulfate on the behavior of paired rats in a competitive situation. Psychol. Rec., 1962, 12, 25-34.

1003539033

(With N. B. Louis and A. Young) Survey of operational rotary wing aviators flying activities. HumRRO Tech. Rep. #75, 1962.

(With N. B. Louis and A. Young) Survey of operational fixed wing aviators flying activities. HumRRO Tech. Rep. #76, 1962.

Social influence on the response to drugs: II. Chlorpromazine and ironiazid. Psychopharmacologia, 1962, 3, 72-78.

(With A. McDonald) Social influence on the response to drugs: III. Age factors in the response to amphetamine sulfate. Psychopharmacologia, 1962, 3, 212-218.

(With A. McDonald) Social influence on the response to drugs: IV. Stimulus factors. Psychol. Rec., 1962, 12, 327-330.

(With R. T. Davis) A simple recording system for the direct observation technique. Animal Behaviour, 1962, 10, 208-210.

(With T. M. Mast) Effects of prior social experience on amphetamine toxicity in mice. Psychol. Rep., 1962, 11, 809-812.

1963

(With T. M. Mast and L. L. Larrabee) Effects of fatigue on basic processes involved in human operator performance: I. Simple vigilance and target detection. Highway Research Record, 1963, 55, 17-20.

(With T. M. Mast and D. K. Spiegel) The relationship between operator mood and performance in a simulated driving task. Tech. Report No. 2, Human Factors Laboratory, University of South Dakota, 1963.

1964

(With A. L. McDonald) Modification of aggressive behavior of green sunfish with D-Lysergic acid diethylamide. J. Psychol., 1964, 57, 19-23.

(With T. M. Mast and H. F. Jones) The effects of fatigue on performance in a simulated driving task. Tech. Report No. 3, Human Factors Laboratory, University of South Dakota, 1964.

1003539034

(With H. F. Jones) An investigation of the relationship between performance on a "speed anticipation" test and driver performance. Tech. Report No. 4, Human Factors Laboratory, Univ. of South Dakota, 1964.

(With T. M. Mast) Effects of fatigue on vigilance. J. Engineering Psych., 1964, 3(3), 73-79.

(With H. F. Jones) Ability of drivers to make critical passing judgments. J. Engineering Psych., 1964, 3(4), 117-122.

1965

(With A. L. McDonald) Agonistic behavior in several species of fish. Psych. Rep., 1965, 16, 845-850.

(With H. F. Jones) Signal detection as function of location. Tech. Report No. 5, Human Factors Laboratory, Univ. of South Dakota, 1965.

A further investigation of the development of mouse killing in rats. Psychonomic Sci., 1965, 2, 179-180.

(With A. L. McDonald) Social influence on the response to drugs: V. Modification of behavior of non-drugged rats by drugged. Psychopharmacologia, 1965, 8, 174-180.

(With S. Sallee) Effects of early drug treatment on adult dominance behavior in rats. Psychopharmacologia, 1965, 8, 235-240.

1966

(With A. R. DeKock) Effects of sustained performance on differential angular velocity judgments. Tech. Rep. No. 6, Human Factors Laboratory, Univ. of South Dakota, 1966.

1967

(With N. R. Bancroft and A. R. DeKock) Effects of smoking upon sustained performance in a simulated driving task. In H. B. Murphy (Ed.), The effects of smoking on the central nervous system. Ann. N. Y. Acad. Sci., 1967.

(With V. S. Ellingstad) Performance decrement during 15 hours operation of a complex psychomotor task. Tech. Rep. No. 7, Human Factors Laboratory, Univ. of South Dakota, 1967.

1003539035

(With V. S. Ellingstad and A. R. DeKock) Effects of operator mood on performance in a simulated driving task. Perceptual and Motor Skills, 1967, 25, 729-735.

1968

(With D. K. Damkot and N. G. Benson) The effects of silt turbidity on behavior of juvenile largemouth bass and green sunfish. Tech. Paper, Bureau of Sport Fisheries & Wildlife, 1968.

(With A. L. McDonald and D. K. Damkot) Social modification of agonistic behaviour in fish. Animal Behaviour, 1968, 16, 437-441.

(With V. Ellingstad) Estimation of movement as a function of target speed, display distance, and concealment distance. Tech. Rep. No. 7, April, 1968, Human Factors Laboratory, USD.

1969

(With V. Ellingstad) Velocity-time estimation as a function of target speed and concealment extent. Human Factors, 1969, 11, 305-312.

(With J. Nichols and G. Martin) An experimental methodology for analysis of child pedestrian behavior. Pediatrics, 1969, 44, (part 2), 832-838.

1970

Effects of "stress fatigue" on performance in a simulated driving situation. Ergonomics, 1970, 13(2), 209-218.

(With V. Ellingstad) Performance changes during sustained operation of a complex psychomotor task. Ergonomics, 1970, 13(6), 693-705.

(With G. Martin) Perception of hazard by young children. Technical Report, 1970, Human Factors Laboratory, USD.

1971

(With H. D. Warner) Effects of intermittent noise on visual search tasks of varying complexity. Perceptual and Motor Skills, 1971, 32, 219-226.

1003539036

1972

(With H. D. Warner) Effects of noise intensity on visual target-detection performance. Human Factors, 1972, 14(2), 177-181.

(With M. A. Hofmann) Tracking performance with visual auditory, or electrocutaneous displays. Human Factors, 1972, 14(2), 127-134.

1973

(With H. D. Warner) Target-detection performance as a function of noise intensity and task difficulty. Perceptual and Motor Skills, 1973, 36, 439-442.

(With R. Lucas & D. Spiegel) Part-task simulation training of driver's passing judgments. Human Factors (in press).

The effects of smoking on mood change. In W. L. Dunn (Ed.) Smoking behavior: Motives and incentives. Washington, D.C.: V.H. Winston & Sons, 1973.

Publications - Books

(Editor) Injury Control in Traffic Safety. Springfield, Ill.: Charles C. Thomas, 1970.

(With V. S. Ellingstad) Human Behavior: A Systems Approach. Monterey, California: Brooks/Cole Publishing Company, April, 1972.

(With A. L. McDonald) Psychology and Contemporary Problems. Monterey, California: Brooks/Cole Publishing Company, February, 1973.

(With V. S. Ellingstad) Methods in the Study of Behavior. (In production -- to be published by Brooks/Cole Publishing Co.)

1003539037

#883 HUTCHINSON

1003539038

October 26, 1972

Grant Application No. 883

To: The committee comprising Drs. Bing, Cattell and Sommers
Subject: Ronald R. Hutchinson, Ph.D., Kalamazoo State Hospital
Michigan
New application No. 883
"The Blood Pressure Reductant Effects of Nicotine"

History

This is a spontaneous application. Dr. Hutchinson is known to CTR staff through his participation in the St. Martins conference. The request is for \$14,030, for one year only.

Documents Submitted (attached)

1. Application dated September 22, 1972

2. Reprints:

- a) "Effects of Long-term Shock...", Hutchinson, Renfrew and Young, J. Exp. Anal. Beh. 15, 141-166 (1971)
- b) "...Behavioral Influence of Chlorpromazine", Emley and Hutchinson, Life Sciences 11, 43-47 (1972)
- c) "Similar and Selective Actions of Chlorpromazine", Emley and Hutchinson, Proc. APA, 1971, 759-760

3. Manuscript "Effects of Nicotine on Avoidance...", Hutchinson and Emley.

Comment

CTR records indicate that earlier applications by Dr. Hutchinson to AMA-ERF have been denied as follows:

October 1970, "Psychophysiological Aspects of Cigarette Deprivation."
May 1972, "The Laboratory Analysis of the Stress Reluctant and Correlated Abuse Potential of Drugs."

This program has substantial grant support from other sources as shown in the application.

F.W.N.

FWN:jfr
Encl:

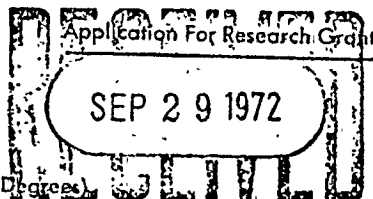
1003539039

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

COMMITTEE:

Dr. Bing
Dr. Cattell
Dr. Sommers

110 EAST 59TH STREET
NEW YORK, N. Y. 10022



Date: September 22, 1972

1. Name of Investigator(s): (include Title and Degrees)

Ronald R. Hutchinson, Ph.D.
Grace S. Emley, M.A.

Director of Research
Research Psychologist

2. Institution &

Address:

Research Department
Kalamazoo State Hospital
Kalamazoo, Michigan 49001

3. Short Title of Project:

The Blood Pressure Reductant Effects of Nicotine

4. Proposed Starting Date:

January 1, 1973

5. Anticipated Duration of this Specific Study: 1 year.

Brief Description of Objectives or Specific Aims:

The objectives of the proposed research are (1) to record the motoric and cardiovascular changes subsequent to stress in animals and man, (2) to study the reductive effects of acute nicotine administration on these reactions.

7. Give a Brief Statement of your Working Hypothesis:

It is proposed to determine if and to what extent nicotine has the therapeutic effect of reducing the cardiovascular changes associated with episodes of stress-produced anger in animals and man.

1003539040

8. Details of Experimental Design and Procedures: (Attach Separate Pages)

See Experimental Protocols #1 and #2 attached

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

Facilities statement attached.

10. Additional Requirements:

1003539041

11. Biographical sketches of all principal and professional personnel (append)
R.R. Hutchinson and G.S. Emley vitae attached.

12. List of publications: (Five most recent as pertinent) (append)

Appendix I

13. Budget: (1st year)

A. Salaries (Personnel by names)

Professional		% time	Amount
	R.R. Hutchinson	5%	REDACTED
	G.S. Emley	5%	
	Research Assistant	100%	

Technical

Engineering - Consultant	25%	REDACTED
--------------------------	-----	----------

Sub-Total

B. Consumable Supplies (list by categories)

Animal care:

Cages, food, bedding, drugs, etc. \$200

Human Subject supplies: Lab coats, electrodes, electrode paste, etc. 120

Misc. Supplies: Recording paper, ink, electrode cabling, alcohol, syringes, antibiotics, etc. 100

Sub-Total

780

C. Other Expenses (itemize)

Graphics & secretarial supplies 100

4 squirrel monkeys @ \$45 180

Human subject compensation 500

Sub-Total

D. Permanent Equipment (itemize)

-0-

12,200

E. Overhead (15% of A+B+C)

1,830

Total

14,030

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2						
Year 3						

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature

Director of Project

Signature

Business Officer of the Institution

Telephone

Telephone

1003539042

Background of proposed research

A. Nicotine has been shown to reduce the emotional effects of an immediately preceding stressor in both animals and man (Emley and Hutchinson, 1971; Hutchinson and Emley, 1971; Emley and Hutchinson, 1972; Hutchinson and Emley, 1972). The overt and covert skeletal muscle responses associated with aggressivity and attack produced by stressors are selectively reduced in their frequency and amplitude subsequent to the administration of nicotine in rats, monkeys and man.

B. An established component of the emotion of anger and/or aggression and attack behaviors is a rapid, though temporary, elevation in blood pressure (Cannon, 1970; Ax, 1953; Hutchinson and Emley, 1972). These reactions are each components in the total attack pattern and are organized in the brain stem. The neural effects of nicotine are thought to be concentrated at the medullary level also (Domino, 1967) and probably influence anger and attack action at these brain sites.

C. Since nicotine is already shown to reduce the behavioral components of anger and the behavioral and cardiovascular effects are known to covary and further, since nicotine is known to have a concentrated action at the neural locus already known to mediate both the behavioral and somatic components of anger, strong evidence thus exists for proposing a blood pressure reductant effect of acute nicotine administration.

D. The above thesis may be reconciled with the frequently observed contrary result of peripherally administered nicotine upon blood pressure, as nicotine is known also to have peripheral vasoconstrictor (and thus blood pressure elevating) effects. Further, the elevated blood pressures sometimes reported for chronic cigarette smokers might well only be a correlational association for a continuing behavioral tendency toward nicotine use caused by a need to reduce anger and hostility. The anger conditions may, in fact, be the cause of both the cigarette consumption and the blood pressure elevation, rather than the nicotine causing the blood pressure increases.

1003539043

VITA

Ronald R. Hutchinson
 Director of Research
 Kalamazoo State Hospital
 Kalamazoo, Michigan 49001

REDACTED

EDUCATION

1. 4 year diploma, General Motors Institute; Majors: Mechanical Engineering, Business Administration. Specialization, Personnel, Research and Administration R
2. M.A., Southern Illinois University, Rehabilitation Counseling-Clinical Psychology, R
3. M.S., Yale University, Experimental Psychology, R
4. Ph.D., Yale University, Experimental Psychology, R

EXPERIENCE

1972-present

Director of Research Kalamazoo State Hospital,
 Kalamazoo, Michigan 49001.

1968-1972

Director of Research, Fort Custer State Home,
 Augusta, Michigan 49012.

1970-present

Adjunct Associate Professor, Psychology Department,
 Western Michigan University. Direct research
 programs in studies of environmental and physiolog-
 ical causes of emotional behavior and teach instru-
 mentation, physiological psychology, motivation
 and emotion.

1967-1970

Associate Professor, as above.

1966-1967

Assistant Professor, as above.

1966-1968

Adjunct Associate Professor, Southern Illinois
 University. Directed Doctoral Candidates.

1963-1966

Adjunct Assistant Professor, Southern Illinois
 University. Directed Doctoral Candidates, taught
 Introductory Psychology and Aversive Control.

1962-1966

Medical Research Associate, Behavior Research
 Laboratory, Anna State Hospital. Coordinated and
 directed research programs on environmental and
 physiological causes of aggressive behavior.

1961-1962

Pre-Doctoral Fellowship, United States Public Health
 Service, Intracranial stimulation, cardiac condition-
 ing salivation, with N.E. Miller, A.R. Wagner,
 and F.P. Gault.

1003539044

R.R. Hutchinson

-2-

Vita

EXPERIENCE (Cont.)

1960-1961

Research Assistant, N.E. Miller, Yale University.
Infra-human research on physiological and environmental influences on behavior.

1959-1960.

Research Associate, Behavior Research Laboratory,
Anna State Hospital. Research in learning and
performance of hospital patients, infra-human
research with N.H. Azrin and W.C. Holz.

MEMBERSHIP IN PROFESSIONAL ORGANIZATIONS, HONORARY SOCIETIES, COMMUNITY ORGANIZATIONS, ETC.

REDACTED

REDACTED

MAJOR RESEARCH INTERESTS

The laboratory analysis of behavior. The study of emotional behavior.

SELECTED PUBLICATIONS

Hutchinson, R.R. and Azrin, N.H. Conditioning of mental-hospital patients to fixed-ratio schedules of reinforcement. J. exp. Anal. Behav., 1961, 4, 87-95.

Azrin, N.H., Hutchinson, R.R. and Hake, D.F. Pain-induced fighting in the squirrel monkey. J. exp. Anal. Behav., 1963, 6, 620.

Azrin, N.H., Ulrich, R.E., Hutchinson, R.R., and Norman, D.G. Effect of shock duration on shock-induced fighting. J. exp. Anal. Behav., 1964, 7, 9-11.

Azrin, N.H., Hutchinson, R.R., and Sallery, R.D. Pain-aggression toward inanimate objects. J. exp. Anal. Behav., 1964, 7, 223-228.

Azrin, N.H., Hake, D.F., Holz, W.C., and Hutchinson, R.R. Motivational aspects of escape from punishment. J. exp. Anal. Behav., 1965, 8, 31-44.

Azrin, N.H., Hake, D.F., and Hutchinson, R.R. Elicitation of aggression by a physical blow. J. exp. Anal. Behav., 1965, 8, 55-57.

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SELECTED PUBLICATIONS (Cont.)

Azrin, N.H., Hutchinson, R.R., and McLaughlin, R. The opportunity for aggression as an operant reinforcer during aversive stimulation, J. exp. Anal. Behav., 1965, 8, 171-180.

Hutchinson, R.R., Ulrich, R.E., and Azrin, N.H. Effects of age and related factors on the pain-aggression reaction. J. comp. physiol. Psychol., 1965, 59, 365-369.

Ulrich, R.E., Hutchinson, R.R., and Azrin, N.H. Pain-elicited aggression. Psychol. Rec., 1965, 15, 111-126.

Azrin, N.H., Hutchinson, R.R., and Hake, D.F. Extinction-induced aggression. J. exp. Anal. Behav., 1966, 9, 191-204.

Hutchinson, R.R., Azrin, N.H., and Hake, D.F. An automatic method for the study of aggression in squirrel monkeys. J. exp. Anal. Behav., 1966, 9, 233-237.

Hutchinson, R.R., and Renfrew, J.W. Stalking attack and eating behaviors elicited from the same sites in the hypothalamus. J. comp. physiol. Psychol., 1966, 61, 360-367.

Azrin, N.H., Hutchinson, R.R., and Hake, D.F. Attack avoidance and escape reactions to aversive shock. J. exp. Anal. Behav., 1967, 10, 131-148.

Hutchinson, R.R. and Renfrew, J.W. A simple histological technique for localizing electrode tracks and lesions within the brain. J. exp. Anal. Behav., 1967, 10, 277-280.

Hutchinson, R.R. and Renfrew, J.W. Modification of eating and drinking: interactions between chemical agent, deprivation state, and site of stimulation. J. comp. physiol. Psychol., 1967, 63, 408-416.

Azrin, N.H. and Hutchinson, R.R. Conditioning of the aggressive behavior of pigeons by a fixed-interval schedule of reinforcement. J. exp. Anal. Behav., 1967, 10, 395-402.

Hutchinson, R.R., Azrin, N.H., and Renfrew, J.W. Effects of shock intensity and duration on the frequency of biting attack by squirrel monkeys. J. exp. Anal. Behav., 1968, 11, 83-88.

Hutchinson, R.R., Azrin, N.H., and Hunt, G.M. Attack produced by intermittent reinforcement of a concurrent operant response. J. exp. Anal. Behav., 1968, 11, 489-495.

Azrin, N.H., Rubin, H.B., and Hutchinson, R.R. Biting attack by rats in response to aversive shock. J. exp. Anal. Behav., 1968, 11, 633-639.

Hutchinson, R.R. Aggression without victims: An investigative approach. Michigan Mental Health Research Bulletin, Winter, 1970, 4, 32-34.

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SELECTED PUBLICATIONS (Cont.)

DeFrance, J., and Hutchinson, R.R. Electrical activity of the amygdala and hippocampus during biting. Michigan Mental Health Research Bulletin, Summer, 1970, 4, 28-30.

Emley, G.S., Hutchinson, R.R., and Brannan, I.B. Aggression: effects of acute and chronic morphine. Michigan Mental Health Research Bulletin, Fall, 1970, 4, 23-26.

Hutchinson, R.R., Renfrew, J.W., and Young, G.A. Effects of long-term shock and associated stimuli on aggressive and manual responses. J. exp. Anal. Behav., 1971, 15, 141-166.

Emley, G.S., Hutchinson, R.R., Hallin, E.R., and Kiraley, S. A convenient method for physical storage of cumulative records. J. exp. Anal. Behav., 1971, 15, 248.

DeFrance, J., and Hutchinson, R.R. Epileptiform activity in the basolateral amygdala related to aggression. Michigan Mental Health Research Bulletin, Winter, 1971, 5, 36-38.

Emley, G.S., Hutchinson, R.R., and Hunter, N.A. Selective actions of morphine, chlorpromazine, chlordiazepoxide, nicotine and d-amphetamine on shock-produced aggressive and other motor responses in the squirrel monkey. Federation Proceedings, 1971, 30, 390.

Hutchinson, R.R., and Emley, G.S. The behavioral basis of action of tranquilizers. Michigan Mental Health Research Bulletin, Spring, 1971, 5, 19-22.

Emley, G.S., and Hutchinson, R.R. Similar and selective actions of chlorpromazine, chlordiazepoxide, and nicotine on shock-produced aggressive and anticipatory motor responses in the squirrel monkey. Proceedings, 79th Annual Convention, APA, 1971, 759-760.

Emley, G.S., and Hutchinson, R.R. Basis of behavioral influence of chlorpromazine. Life Sciences, 1972, 11, 43-47.

Hutchinson, R.R., and Emley, G.S. Schedule-independent factors contributing to schedule-induced phenomena. In R.M. Gilbert and J.D. Keehn (eds), Schedule Effects Drugs, Drinking, and Aggression, Toronto: University of Toronto Press, 1972, 174-202.

Hutchinson, R.R. and Emley, G.S. Effects of nicotine on avoidance, conditioned suppression and aggression response measures in animals and man. Conference on Motivation in Cigarette Smoking. Council for Tobacco Research, Academic Press, to be published Fall, 1972.

Hutchinson, R.R. Environmental causes of aggression. Nebraska Symposium on Motivation, 1972, U. of Nebraska Press, to be published, 1973.

1003539047

VITA

Grace S. Emley
Research Psychologist
Kalamazoo State Hospital
Kalamazoo, Michigan 49001

Born:

REDACTED

EDUCATION

M.A. Western Michigan University, Kalamazoo, Michigan, Experimental Psychology, August R

A.B. Chatham College, Pittsburgh, Pennsylvania, Psychology, R

EXPERIENCE

1972-present

Research Psychologist, Research Department,
Kalamazoo State Hospital.

1969-1972

Research Psychologist at Research Department,
Fort Custer State Home, Augusta, Michigan.
Working in research on the effects of pharmacological agents on aggressive behavior.

1968-1969

Graduate Research Assistant with Dr. R.R. Hutchinson, Dept. Psychology, W.M.U. Conducted research on the effects of stimulant and depressant drugs on aggression in the squirrel monkey.

Winter 1968

Computer Programmer, Dr. H. Mitzel, Dept. of continuing Education, Pennsylvania State University. Wrote course material and computer program for a course in medical technology in cooperation with Bethesda Naval Medical School.

1964-1967

Research Assistant to Dr. B.R. Lucchesi, Dr. C.R. Schuster, U. of Michigan Medical School, Ann Arbor, Michigan. Conducted research with humans on the psychological and pharmacological effects of cigarette smoking.

1963-1964

Student assistant to Dr. O.S. Ray, Veterans Administration Hospital, Pittsburgh, Pennsylvania. Conducted research for undergraduate thesis.

1003539048

Publications

Ray, O.S., and Emley, G.S. Time factors in interhemispheric transfer of learning. Science, 1964, 144, 76-78.

Ray, O.S., and Emley, G.S. Interhemispheric transfer of learning. Life Sciences, 1965, 4, 271-279.

Lucchesi, B.R., Schuster, C.R., and Emley, G.S. The role of nicotine as a determinant of cigarette smoking frequency in man with observations of certain cardiovascular effects associated with the tobacco alkaloid. Clinical Pharmacology and Therapeutics, 1967, 8, 789-796.

Emley, G.S., Schuster, C.R., and Lucchesi, B.R. Trends observed in the time estimation of three stimulus intervals within and across sessions. Perceptual and Motor Skills, 1968, 26, 391-398.

Schuster, C.R., Lucchesi, B.R., and Emley, G.S. The effects of certain pharmacological agents on the cigarette smoking frequency in man. Paper presented at the AMA-ERF Session on Research on Tobacco and Health, San Francisco, June 19, 1968.

Emley, G.S. The effects of morphine on shock-induced aggression in the squirrel monkey. Unpublished Masters thesis, Western Michigan University, August, 1969.

Emley, G.S., Hutchinson, R.R., and Brannan, I.B. Aggression: effects of acute and chronic morphine. Michigan Mental Health Research Bulletin, Fall, 1970, 4, 23-26.

Emley, G.S., Hutchinson, R.R., Hallin, E.R., and Kiraley, S. A convenient method for physical storage of cumulative records, J. exp. Anal. Behav., 1971, 15, 248.

Emley, G.S., Hutchinson, R.R., and Hunter, N.A. Selective actions of morphine, chlorpromazine, chlordiazepoxide, nicotine, and d-amphetamine on shock-produced aggressive and other motor responses in the squirrel monkey. Federation Proceedings, 1971, 30, 390.

Hutchinson, R.R., and Emley, G.S. The behavioral basis of action of tranquilizers. Michigan Mental Health Research Bulletin, Spring, 1971, 5, 19-22.

Emley, G.S., and Hutchinson, R.R. Similar and selective actions of chlorpromazine, chlordiazepoxide, and nicotine on shock-produced aggressive and anticipatory motor responses in the squirrel monkey. Proceedings, 79th Annual Convention, APA, 1971, 759-760.

Hutchinson, R.R., Emley, G.S. and Sauer, R.A. Effects of cessation of cigarette smoking on jaw clenching frequency in humans. Paper presented at APA, Washington, D.C., 1971.

Emley, G.S. and Hutchinson, R.R. Basis of behavioral influence of chlorpromazine, Life Sciences, 1972, 11, 43-47.

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Publications (Cont.)

Hutchinson, R.R. and Emley, G.S. Schedule-independent factors contributing to schedule-induced phenomena. In R.M. Gilbert and J.D. Keehn (eds), Schedule Effects: Drugs, Drinking, and Aggression, Toronto, University of Toronto Press, 1972, 174-202.

Hutchinson, R.R. and Emley, G.S. Effects of nicotine on avoidance, conditioned suppression and aggression response measures in animals and man. Conference on Motivation in Cigarette Smoking. Council for Tobacco Research, Academic Press, to be published, Fall, 1972.

1003539050

Experimental Protocol #1

Subjects:

Two naive adult squirrel monkeys weighing 900 to 1100 grams will be used. Subjects will be maintained on ad libitum food and water throughout the experimental series.

Procedure:

Subjects will be implanted with chronic indwelling cannulae inserted through an incision in the jugular vein in the neck. The distal tip of the cannulae will extend to approximately one-half centimeter above the aortic arch. The methods have already been perfected for a long term monitoring of blood pressure in the squirrel monkey (Herd, Morse Kelleher and Jones, 1968). The exposed tip of the cannulae will be connected to a Statham pressure transducer during recording sessions. Subjects will be tested daily for one hour sessions on programs of response-independent electric shocks delivered to the tail. Shock will be delivered each four minutes. Previous work has shown that a flurry of biting responses will occur immediately subsequent to shock (Hutchinson, Azrin and Hake, 1966). Additionally, it has been shown that subsequent to shock, blood pressure increases will occur (Ferreira, Gollub and Vane, 1969). These conditions will be maintained until response stability is present. Subsequently, subcutaneous doses of nicotine tartrate will be administered in a random dose response procedure. Procedural details of drug administration, dosage, etc., will be as in Hutchinson and Emley (1972). The dependent measures will be correlations between attack and blood pressure elevations and the reduction in these effects as a function of nicotine administration.

1003539051

Experimental Protocol #2.

Subjects:

Five adult male human volunteers will serve as subjects.

See Appendix 2 for human subject treatment procedures.

Procedure:

Prior to each experimental session, subjects will be prepared for the recording of heart rate, blood pressure, peripheral vasomotor action (vasoconstriction of the left index finger), and contraction of masseter and temporalis muscle of the forehead. Blood pressure will be continuously monitored at the radial artery by the method of constant volume, pressure measurements, already perfected by Pressman and Newgard (1963). Subjects will be seated in a sound-attenuated space and exposed to periodic delivery of pure tones each four minutes. Tone intensity will be 110 db, lasting for 2 seconds. Subsequent to stabilization of baseline conditions, subjects will be given several dosages of nicotine tartrate orally in five ounces of pure distilled water. Dosage will range from 2 to 10 mg. and pre-treatment will be 15 minutes prior to actual testing. The observation of principal interest in this study will be to determine whether nicotine produces a reduction in cardiovascular responses to intense noises similar to the reduction already observed and reported for jaw clenching responses subsequent to noxious stimuli. (Hutchinson and Emley, 1972).

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References

- Ax, A.F. The physiological differentiation between fear and anger in humans. Psychosom. Med., 1953, 15, 433-442.
- Cannon, W.B. Bodily changes in pain, hunger, fear and rage. Maryland: McGrath, 1970, 93.
- Domino, E.F. Electroencephalographic and behavioral arousal effects of small doses of nicotine: a neuropsychopharmacological study. Ann. New York Acad. Sci., 1967, 142, 216-244.
- Emley, G.S. and Hutchinson, R.R. Similar and selective actions of chlorpromazine, chlordiazepoxide, and nicotine on shock-produced aggressive and anticipatory motor responses in the squirrel monkey. Proceedings, 79th Annual Convention, APA, 1971, 759-760.
- Emley, G.S. and Hutchinson, R.R. Basis of behavioral influence of chlorpromazine. Life Sciences, 1972, 11, 43-47.
- Ferreira, S.H., Gollub, L.R., and Vane, J.R. The release of catecholamines by shocks and stimuli paired with shocks. J. exp. Anal. Behav., 1969 12, 623-631.
- Herd, J.A., Morse, W.H., Kelleher, R.T. and Jones, L.G. Arterial blood pressure in the squirrel monkey during behavioral experiments. Fed. Proc., 1968, 27, 743.
- Hutchinson, R.R., Azrin, N.H., and Hake, D.F. An automatic method for the study of aggression in squirrel monkeys. J. exp. Anal. Behav., 1966, 9, 233-237.
- Hutchinson, R.R. and Emley, G.S. The behavioral basis of action of tranquilizers. Michigan Mental Health Research Bulletin, Spring, 1971, 5, 19-22.
- Hutchinson, R.R. and Emley, G.S. Effects of nicotine on avoidance, conditioned suppression and aggression response measures in animals and man. Conference on Motivation in Cigarette Smoking. Council for Tobacco Research, Academic Press, to be published Fall, 1972.
- Pressman, G.L. and Newgard, P.M. A transducer for the continuous external measurement of arterial blood pressure. IEEE Transactions on Bio-Medical Electronics, 1963, April, 73-81.

1003539053

Facilities

1. Space: Approximately 16,680 square feet.

2. Major Facilities and Equipment:

- a. Equipment fabrication and repair shop: Contains full complement of hand and power tools, standing power machinery (lathes, vertical mill, table and band saws, grinder, etc.).
- b. Electronic testing and repair room: 180 square feet of workbench, with full complement of electronic components and testing equipment.
- c. Animal colony room: Contains food and medication storage, cages, scales, handling benches. Heated and air-conditioned for constant temperature control.
- d. Experiment control room: Includes automated control, monitor, and recording equipment for animal test chambers; program-panel cabinets; files for records; desks, etc., for operators.
- e. Two animal test-chamber rooms: Each contains five large isolation test chambers remotely wired to control room. Air-conditioned for constant temperature control.
- f. Human test chamber rooms: Three Industrial Acoustic Corp. chamber rooms, each within their own individual rooms and connected to a control and monitor room.
- g. Histology lab: Contains cabinets, formica counters, sink, includes space for microtome, analytical balance, microscope, drug refrigerator, drug safe, and appropriate glassware and supplies.
- h. Experimental surgeries (2): Includes operating tables, cabinets, stimulators, polygraphs, oscilloscopes, respirators, stereotaxes, and appropriate supplies and instruments.
- i. Drafting room and Darkroom: Includes two enlargers, print dryer, developing and printing tank, film dryer, and two drafting tables and stools.
- j. Library/conference room: Includes conference table, chairs, periodical racks, reprint files, photocopier, and approximately 500 reference volumes pertinent to physiology and psychology.
- k. Offices (8): Contain usual complement of office furniture and equipment for researchers and secretaries.

1003539054

Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current

Title of Project	Source	Amount	Duration
Environmental Causes of Aggression	National Science Foundation	\$51,810	4/72 - 4/75
Causes and Control of Aggression in Man	Office of Naval Research	\$37,957	3/72 - 2/73
Measurement and Facilitation of Cooperative Task Performance	National Aeronautics and Space Administration	\$36,000	8/72 - 2/73

Pending

1003539055

APPENDIX I

1003539056

Publications appended.

Hutchinson, R.R., Renfrew, J.W., and Young, G.A. Effects of long-term shock and associated stimuli on aggressive and manual responses. J. exp. Anal. Behav., 1971, 15, 141-166.

Emley, G.S. and Hutchinson, R.R. Similar and selective actions of chlorpromazine, chlordiazepoxide, and nicotine on shock-produced aggressive and anticipatory motor responses in the squirrel monkey. Proceedings, 79th Annual Convention APA, 1971, 759-760.

Hutchinson, R.R., Emley, G.S. and Sauer, R.A. Effects of cessation of cigarette smoking on jaw clenching frequency in humans. Presented at APA, 1971.

Emley, G.S., and Hutchinson, R.R. Basis of behavioral influence of chlorpromazine. Life Sciences, 1972, 11, 43-47.

Hutchinson, R.R. and Emley, G.S. Effects of nicotine on avoidance, conditioned suppression and aggression response measures in animals and man. Conference on Motivation in Cigarette Smoking. Council for Tobacco Research Academic Press, to be published, Fall, 1972.

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APPENDIX 2

1003539058

Treatment of Human Subjects

Prior to implementation of any human experimental procedures, they are reviewed in detail by the Institution's Ethics Committee. Measures taken to protect all human subjects include pre- and post- experimental medical examinations, documentation of subject consent and briefing, and subject's knowledge that he is free to withdraw from any experiment at any time.

Subjects for these studies will be asked to complete a medical history questionnaire. Each subject will then be given a complete physical exam before the experiments begin. Ralph M. Hodges, M.D., (Kalamazoo, Michigan) will be giving the physical exams. The examinations will consist of hemoglobin, and urinalysis tests in addition to a general physical exam. Parts of the examination will be repeated at the conclusion of the experiment.

Before each experimental session, the subjects will sign a release form giving their consent to serve in the experimental procedure. The subjects are also protected under the state and institution insurance policy which covers any injury or accident incurred while on the grounds of the Kalamazoo State Hospital. At the end of the experimental session, the subject will sign another form which indicates that he has served in the experiment.

All experiments will be conducted with the subject residing in a sound-attenuated, electrostatically shielded, 6 foot by 6 foot room. There will be full-time audio and visual monitoring of the subject within the chamber, and full-time monitoring of cardiac and behavioral processes by the subjects. A physician will be available at all times and all laboratory personnel will be instructed in normal first aid procedures, including cardiac massage and artificial respiration. Resting cots, smelling salts and blankets are always available as medical support apparatus.

Compounds will be administered by medical personnel. A physician will be in residence at all times during these experiments and all subjects will remain under observation until a physician is sure that all drug effects have dissipated or diminished to a negligible extent and will not interfere with normal functioning. Additionally, subjects will be transported to their place of residence subsequent to this time by laboratory staff and equipment.

Subjects will be paid for all services. Pay will be approximately \$5.00 per experimental session, paid at the completion of each session, with a pay bonus to be given at the end of the entire experimental series. The pay bonus will accumulate in an account for each subject and will be earned at a constant rate per session dependent upon regular, prompt daily attendance.

Analysis of results will be as described in previous experiments to determine increases and decreases in post- or pre-noxious event responses and heart rate. Again, though the mood adjective check list and temperament survey do not constitute primary measures in this experiment, they are added as the response cost to both subject and experimenter is minimal, and the potential extension of response generality and impact in clinical circles is high.

1003539059

NAME _____ AGE _____ SEX _____
ADDRESS _____ PHONE _____

DATE OF BIRTH _____ MARITAL STATUS _____

OCCUPATION _____

ARE YOU A UNITED STATES CITIZEN? YES _____ NO _____

Medical history:

1. Do you consider yourself to be in good health at the present time?

yes--- _____ no _____

2. Have you ever been refused employment, insurance, or rejected from the
armed forces because of your health? yes _____ no _____

3. Do you now or have you ever had any of the following illnesses?

	Yes _____	NO _____
asthma	_____	_____
bronchitis	_____	_____
hay fever	_____	_____
rheumatic fever	_____	_____
epilepsy	_____	_____
tuberculosis	_____	_____
diabetes	_____	_____
increased blood pressure	_____	_____
stomach trouble	_____	_____
migraine headache	_____	_____
serious infections	_____	_____
skin trouble	_____	_____
arthritis	_____	_____
tooth ache	_____	_____
bleeding gums	_____	_____
bladder or kidney trouble	_____	_____
nervous or mental disorder	_____	_____
infectious mononucleosis	_____	_____
heart trouble	_____	_____

4. Do you have any allergies to drugs, foods, animals, plants, insects, dust, etc?

yes _____ no _____ If yes, please explain.

1003539060

5. Are you presently under the care of a physician, psychiatrist or thearapist?

yes _____ no _____

6. Are you presently or have you recently received any medication for any reason
(including vitamins, pep pills, diet pills, tranquilizers, etc.)?

yes _____ no _____

7. Are you on any special diet? yes _____ no _____

8. Has anyone in your family had any of the following illnesses? (grandparents,
parents, brothers, sisters, children)

	yes _____	no _____	Relationship _____
asthma	_____	_____	_____
hay fever	_____	_____	_____
epilepsy	_____	_____	_____
nervous or mental disorder	_____	_____	_____
cancer	_____	_____	_____
heart disease	_____	_____	_____
severe headache	_____	_____	_____
high blood pressure	_____	_____	_____

9. When was your last physical exam?

10. When did you last have a dental check up?

GENERAL INFORMATION

1. Have you ever taken part in any experimental studies? Yes _____ No _____
If yes, did these studies involve any of the following:

	Yes _____	No _____
ingestion of food substances	_____	_____
ingestion or injection of drugs	_____	_____
metabolic tests	_____	_____
blood or urine tests	_____	_____
blood pressure recording	_____	_____
abstinence of any sort	_____	_____
heart rate recording	_____	_____
EEG recording	_____	_____
confinement	_____	_____
physical exertion tests	_____	_____
written tests	_____	_____
performance tests	_____	_____
sleep	_____	_____

1003539061

2. Are you right handed? yes _____ no _____

3. Do you wear any of the following:

False teeth or Bridge

yes _____ no _____

Hearing aid

Glasses

Braces for teeth

4. Have you ever been bothered by claustrophobia? yes _____ no _____

5. Are you currently enrolled in an educational program of any sort?

yes _____

no _____

6. Please indicate daily or weekly frequency of the following?

a. Sleep (hrs.)

b. Alcohol consumption

c. Exercise (hrs.)

d. Tobacco consumption (cigars, cigarettes, pipe)

e. Chewing gum

f. Coffee

g. Tea

7. Do you suffer from headaches?

yes _____ no _____

8. Are you a nail biter or pencil chewer?

yes _____ no _____

9. Do you participate actively in sports?

yes _____ no _____

Signed _____

Witness _____

Date _____

1003539062

Consent to Experimental Procedure

I, _____ hereby consent to serve as a volunteer
experimental subject for the Research Department of the Kalamazoo State
Hospital. I have been informed of the experimental procedure. I
understand that I may withdraw at anytime from participation in this
research.

Signed _____

Witness _____

Date _____

1003539063

Release From Experimental Procedure

I, _____ have served as a volunteer experimental
subject for the Research Department of the Kalamazoo State Hospital.

I felt _____, did not feel _____, unpleasant side effects. If
so they were _____

Signed _____

Witness _____

Date _____

1003539064

#881 MISTELIN

1003539065

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

November 6, 1972

Grant Application No. 881

5
Denied

To: The committee comprising Drs. Andervont, Huebner, and Sommers
Subject: Victor Milstein, Ph.D., Indiana University Medical Center,
Indianapolis, Indiana
New application No. 881
"Tobacco, Reinforcers and Clinical Status"

History

This proposal was Case No. 119, and full application was encouraged by the Planning Committee.

Application No. 881 requests \$6,843, for one year only.

Documents Submitted (attached)

1. Application dated September 26, 1972.
2. As the application is somewhat terse, staff has obtained the following supplementary information:
 - a. Letter from Milstein dated October 9, 1972.
 - b. Copies of the six Rating Scales mentioned in item 8 of the application.
 - c. Reprint of Milstein's most recent publication, mentioned in item 12 of the application.
 - d. "The Token Economy System: An Introduction and Explanation for Patients and Other Interested Persons".

Comment

Staff has obtained an evaluation from Kurt Salzinger, Ph.D., principal Research Scientist, New York State Department of Mental Hygiene. Attached is a copy of Salzinger's letter dated October 30.

1003539066

F.W.N.

FWN:wg
Encls.



ALAN D. MILLER, M.D.
COMMISSIONER

STATE OF NEW YORK
DEPARTMENT OF MENTAL HYGIENE

JOSEPH ZUBIN, PH.D.
CHIEF OF PSYCHIATRIC RESEARCH
(BIOMETRICS)

BIOMETRICS RESEARCH

722 WEST 168 STREET, NEW YORK N. Y. 10032
LORRAINE 8-4000 • WADSWORTH 7-8463

October 30, 1972

Dr. Frederic W. Nordsiek
Associate Scientific Director
The Council for Tobacco Research
110 East 59th Street
New York, New York 10022

Dear Dr. Nordsiek:

I should say at the outset that the enclosed proposal is singularly unimpressive. Although the investigators say that they have a great deal of experience in behavior modification, I see nothing in their Vitae to demonstrate that. Nor is there anything in the listed publications to give one the feeling that they have worked in this area.

You described the study to me as a fishing expedition and indeed it is. Presumably smokers and nonsmokers differ reliably from each other on a number of variables - if you consider large enough groups of subjects. When examining as few as 10 subjects to personify nonsmokers, however, the number of other, extraneous variables which could possibly explain results requires that the investigators give a rather detailed description of the patients in terms of such obvious variables as age, sex, time of onset of disorder, physiological complications, etc. In other words, we should have some basic information on the two groups of patients so that we can at least approach attributing whatever differences are found to the smoking variable (if one can call that one variable).

Now I would expect them to find a difference between the two populations on the basis that smokers find the receipt of cigarettes reinforcing and therefore are particularly susceptible to the conditioning effects of a token economy. Presumably if you contrasted two populations differing on the degree to which they eat and ask for candy, you should get the same kind of difference there. What I am saying is that at its best such a study would show that if you can give patients items that they particularly will work for, then their behavior will change. But this is, of course, the general object of token economies and of behavior modification in general. What one does in such a situation is to find for each patient those items he will most work for. Those are then used as positive reinforcers.

(No. 881)

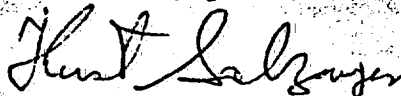
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Perhaps the one factor in favor of this proposal is the small amount of money requested. It will probably give the investigators an opportunity to evaluate the effectiveness of the procedures they are using anyway, although they apparently will have no control as to who gets drugs, what kind, and in what dosage, therefore again questioning the usefulness of their results.

Finally, I should make a comment on the rating scales. Some of them sound vaguely familiar to me. All of them in any case require that the investigators show that their use of these scales is reliable, that is, at least some of the patients should be rated by more than one investigator separately and the ratings then compared. I would have much more trust in the behavioral observations which are ordinarily done in token economies, for these observations are quantifiable in terms of frequency of occurrence of the various types of target behaviors.

I hope that you find this evaluation helpful.

Sincerely yours,



KURT SALZINGER, Ph.D.
Principal Research Scientist

KS:rc
Enc.

1003539068

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

COMMITTEE:

Dr. Andervont
Dr. Huebner
Dr. Sommers

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

Application For Research Grant

OCT 2 1972

Date: September 26, 1972

1. Name of Investigator(s): (include Title and Degrees)

Victor Milstein, Ph.D., Psychophysiological & Associate Professor of Psychiatry/Psychology.
Joyce G. Small, M.D., Director of Research & Laboratories and Professor of Psychiatry.

2. Institution &

Address:

Larue D. Carter Memorial Hospital and Indiana University Medical Center,
1315 West Tenth Street, Indianapolis, Indiana 46202.

3. Short Title of Project:

Tobacco, reinforcers and clinical status.

4. Proposed Starting Date:

April 1, 1973

5. Anticipated Duration of this Specific Study:

One year.

6. Brief Description of Objectives or Specific Aims:

The purpose of this investigation is to examine in a systematic fashion clinical status, somatic treatment, cigarette smoking and adjustment to a ward's token economy. The two specific objectives are:

- (1) To divide the resident population of the token economy research ward into a group of smokers and a group of non-smokers, and to compare these two groups in terms of their response to the ongoing behavior modification treatment program which the ward features.
- (2) To examine changes in behavior on the ward and changes in smoking patterns along with observations relating to the patient's status during treatment with drugs for the underlying psychiatric problem as well as during those times when no drug therapy is ~~not~~ being employed.

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7. Give a Brief Statement of your Working Hypothesis:

On the basis of informal and preliminary observations we would predict that (cont')

8. Details of Experimental Design and Procedures: (Attach Separate Pages)

Patients will be divided into two groups, the group of approximately 30 smokers and a group of about 10 non-smokers. The former will be further divided and categorized in terms of the amount they smoke. A number of very well standardized, reliable observational techniques with which we have a great deal of experience will be used to rate the patients on a weekly basis. If changes are occurring, the frequency of ratings will be increased. These include:

- (1) Brief Psychiatric Rating Scale
- (2) Clinical Global and Improvement Scale
- (3) Nurses Observation Scale of Inpatient Evaluation
- (4) Treatment of Emergent Symptoms
- (5) Patient Personnel Data Inventory
- (6) Symptom Rating Scale

Scales 1 and 2 are instruments filled out by the ward psychiatrist; 3, 4, and 5 by the research nurse; and 6 is a subjective rating scale filled out by the patient. In addition, observations and recordings are made on the research ward. These include a daily recording of work performance, personal grooming, keeping room clean, and performing the various ward duties which are assigned to the individual patients. Since the research ward is run as a token economy many of the privileges of the patients must be purchased and an accurate record is kept of these purchases. The privileges include cigarettes, a visit to the snack bar, coffee, a room to sleep in (cont')

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

Larue D. Carter Memorial Hospital, where the research will be conducted, is an acute intensive care facility under the Mental Health Department of the State of Indiana. It is the teaching and research facility admitting a wide variety of patients from throughout the state. One ward had been set aside and designated as the "research (cont')

10. Additional Requirements:

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Biographical sketches of all principal and professional personnel (append)

See the appended Curriculum Vitae.

12. List of publications: (Five most recent as pertinent) (append)

See Curriculum Vitae (cont')

13. Budget (1st year)

A. Salaries (Personnel by names)

Professional

% time

Amount

Technical

Contracted clerical/typing services.

\$3600.00

Sub-Total

B. Consumable Supplies (list by categories)

Patient comforts -

Observation and Test Forms -

1500.00

150.00

1650.00

Sub-Total

C. Other Expenses (itemize)

IBM Card Punching and Computer Assistance -
Travel -

450.00

250.00

700.00

Sub-Total

D. Permanent Equipment (itemize)

E. Overhead (15% of A + B + C)

892.50

Total

\$6842.50

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2						
Year 3						

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature

Director of Project

634-8401 Ex 318

Telephone

Signature

Business Officer of the Institution

Iver F. Small, M.D.

for D. F. Moore, M.D.

Telephone

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Other Sources of Financial Support

List financial support for research from all sources, including own Institution, for this and/or related research projects.

Current

Title of Project

Source

Amount

Duration

The State of Indiana supports research efforts at

Larue D. Carter Memorial Hospital.

Pending

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7. cont'

patients who are smokers and for whom tobacco reinforcers are available, do better in the token economy of the research ward than patients for whom such reinforcers are not available. With respect to the correlations we are seeking, we expect that alterations in smoking behavior will be indicative of alterations in the patient's general status and will correlate with the various ratings that will be made of the patients.

8. cont'

as opposed to the open ward, movies outside of the hospital, etc.

The analysis of the data involves the construction of correlation matrices among these standardized observations and rating scales. This correlation matrix will be further examined by means of a Factor Analysis which will permit an assessment of the interrelations among all of these observations including that of tobacco use. A separate Factor Analysis will be conducted over the correlations for the group of patients who smoke to determine the influence of amount of tobacco consumption. It is estimated that it will require approximately 10 months to accumulate all the data and 2 months to analyze it.

9. cont'

ward". A token economy, behavior modification approach is currently utilized on this ward, and has been for more than three years. All of the nursing personnel on the ward are familiar with the concepts relating to token economies, often making important suggestions leading to alterations of individual behavior modification programs.

12. cont'

Milstein, V. and Small, J.G., Psychological correlates of 14 and 6 positive spikes, 6/sec spike-waves and small sharp spike transients. Clin. Electroenceph. 2:206-212, 1971.

Milstein, V., Small, J.G. and Small, I.F., The subtraction of serial sevens test in psychiatric patients. Arch. Gen. Psychiat., 26:439-441, 1972.

Milstein, V., Small, J.G., Gans, G. and Moore, J.E., Risk taking and CNV. Presented at the Amer. EEG Society, Houston, Texas, Oct. 1972.

Small, J.G., and Milstein, V. and Golay, S.J., Clinical EEG findings with covert drug abuse. Presented at the Amer. EEG Society, Houston, Texas, Oct. 1972.

Small, I.F., Milstein, V., Sharpley, P. and Small, J.G., A comparative study of mental illness. Presented at the Society for Neuroscience, Houston, Texas, 1972.

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CURRICULUM VITAE

Full Name Victor Milstein

Date of Birth **REDACTED**

Nationality

Address: Home

Office Larue D. Carter Memorial Hospital
1315 West Tenth Street
Indianapolis, Ind. 46202
(317) 634-8401 Ext. 318**REDACTED**EDUCATIONDEGREE YEAR CONFERREDBrooklyn College, Brooklyn, New York
City College of New York, New York
University of Oregon, Eugene, Oregon**REDACTED**Psychological Externship: Psychopathic Hospital 1952-53
University of Iowa
Iowa City, Iowa

Public Health Service Research Fellow of the N.I.N.D.B. 1959-61

Second Advanced Course in Electroencephalography,
Salzburg, Austria 1965PROFESSIONAL EXPERIENCE

Larue D. Carter Memorial Hospital, Indianapolis, Ind., Psychophysicologist	1970-present
Indiana Univ. Medical Center, Indianapolis, Ind., Assoc. Prof. Psychol. in Psychiatry	1970-present
Univ. of Oregon Medical School, Portland, Asst. Prof. Neurology	1966-1970
Oregon Regional Primate Research Center, Beaverton, Oregon Asst. Scientist (half time)	1963-66
Univ. of Oregon Medical School, Portland, Research Fellow in Neurology	1957-59 & 1961-66
Univ. of Oregon, Eugene, Oregon, Teaching Fellow in Psychology	1956-57
Univ. of Oregon, Eugene, Oregon, Research Asst. in Psychology	1955-56
Univ. of Iowa Hospital, Iowa City, Iowa, Psychologist	1953-54
Boro Hall Academy, Brooklyn, N.Y., Guidance Counselor	1951-52

ORGANIZATIONS**REDACTED**

1003539074

CURRICULUM VITAE

Name: SMALL, Joyce Graham

Date of Birth:

Place of Birth:

REDACTED

Marital Status:

Citizenship:

Education: University of Saskatchewan B.A. (Great Distinction)
Biology and Chemistry

University of Manitoba

University of Michigan
Neurology

REDACTED

Internships and Residencies:

Winnipeg General Hospital, Rotating Internship, June 1, 1955 to May 31, 1956.

Ypsilanti State Hospital, Ypsilanti, Michigan, Resident in Psychiatry, 1956 to 1959.

Fellowships:

Teaching Fellow, Biochemistry, University of Manitoba, 1955 to 1956.

Hospital Positions:

Assistant Chief of Male Service, Ypsilanti State Hospital, 1958 to 1959.

Psychiatric Consultant to Crippled Children's Division, University of Oregon Medical School, 1960 to 1962.

Supervising Psychiatrist, Malcolm Bliss Mental Health Center, February to June, 1962.

Visiting Physician, St. Louis City Hospitals, July, 1962 to June, 1965.

Director of Outpatient Clinic and Consultation Services and Director of EEG Laboratory, Malcolm Bliss Mental Health Center, July, 1962 to March, 1964.

Clinical Director, Malcolm Bliss Mental Health Center, April, 1964 to June, 1965.

Clinical Director of Research and Laboratories, Larue D. Carter Memorial Hospital, June, 1965 -

Attending Staff, Veteran's Administration Hospital, Indianapolis, Indiana, July, 1965 - 1969.

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Teaching Positions:

Teaching Fellow (Biochemistry), University of Manitoba, 1955 to 1956.

Instructor in Psychiatry (EEG), Neuropsychiatric Institute, University of Michigan, 1959 to 1960.

Instructor in Psychiatry, University of Oregon Medical School, 1960 to 1961.

Research Associate, Department of Neurology, University of Oregon Medical School, 1960 to 1962.

Assistant Professor of Psychiatry, University of Oregon Medical School, 1961 to 1962.

Assistant Professor of Psychiatry, Washington University School of Medicine, 1962 to 1965.

Associate Professor of Psychiatry, Indiana University School of Medicine, 1965 to 1969.

Professor of Psychiatry, Indiana University School of Medicine, 1969 -

Membership in Societies:

REDACTED

Certifications:

Certified by American Board of Neurology and Psychiatry in Psychiatry, 1961.

Certificate in Clinical Electroencephalography, Board of Qualification of the American EEG Society, 1963.

Fellow of the American Psychiatric Association, 1966.

State Licenses:

Licentiate of the Medical Council of Canada, 1956
State of Michigan (by examination), 1957
State of Oregon (by examination), 1960
State of Missouri (by reciprocity), 1962
State of Indiana (by reciprocity), 1965

Offices and Appointments

Secretary-Treasurer - Central Association of Electroencephalographers 1967-68
President-Elect - Central Association of Electroencephalographers 1969
President - Central Association of Electroencephalographers 1970
Councilor - Central Association of Electroencephalographers 1971-72
Member - Scientific Program, Committee on American EEG Society Meeting,
Washington, D.C. - September, 1970
Member - American Board of Qualifications in Electroencephalography Inc. 1971-76
Chairman - Program and Local Arrangements - Central Association Electro-
encephalographers - Indianapolis Meeting - April, 1972.
Consultant - NIMH Workshop on Psychobiology of Depression, Williamsburg
April 1969
NIMH
Consultant -/Workshop on Psychobiology of Electroconvulsive Therapy - Puerto
Rico, April, 1972
Member - Clinical Psychopharmacology Research Review Committee of the National
Institute of Mental Health 1972-1976

#879 - MORTIER

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2

October 24, 1972

Grant application No. 879

To: The committee comprising Drs. Andervont, Gardner and Meier

Subject: H. David Mosier, Jr., M.D., University of California,
Irvine
New application No. 879
"Effect of Nicotine on the Rat Fetus"

History

This proposal originally was Case No. 135; full application was encouraged by the Planning Committee.

Application No. 879 requests \$29,513, plus two additional years.

Documents Submitted (attached)

1. Application dated September 18, 1972, with letter to Dr. Hockett of the same date.

2. Letter to Dr. Hockett dated October 5, 1972, with 6-page "Supporting Statement for Application", dated October 4, 1972.

3. Reprint "Effect of Maternal Nicotine Intake...", Mosier and Armstrong, Proc. Soc. Exp. Biol. & Med. 124, 1135-1137 (1967)

4. Manuscript "Distribution and Fate of Nicotine...", Mosier and Jansons, Teratology (in press)

Comment

Dr. Mosier's reasons for requesting an April 1, 1973 starting date, should his application be approved, are given in the third paragraph of his letter to Dr. Hockett dated October 5, 1972.

CTR staff considers funding, by the National Institute of Child Health and Human Development, of Dr. Mosier's approved application to be unlikely; but we shall follow this outcome.

JWM

F.W.N.

FWN:jfr
Encl.

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UNIVERSITY OF CALIFORNIA, IRVINE

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SANTA BARBARA • SANTA CRUZ

DEPARTMENT OF PEDIATRICS
CALIFORNIA COLLEGE OF MEDICINE

IRVINE, CALIFORNIA 92664

Please Reply to:

MEMORIAL HOSPITAL OF LONG BEACH
2801 ATLANTIC AVENUE
LONG BEACH, CALIFORNIA 90801
(213) 598-36

October 5, 1972

Re: Case No. 135

Robert C. Hockett, Ph.D.
Acting Scientific Director
The Council for Tobacco Research - U.S.A., Inc.
110 East 59th Street
New York, New York 10022

Dear Dr. Hockett:

Dr. Nordsiek called me September 27 with reference to our application to the Council for Tobacco Research, U.S.A., Inc. for research support and advised that we send additional supporting material. These specifically were a justification of our budget and a statement on the relevance of the proposed work to research in tobacco and health. This information is provided on separate sheets enclosed with this letter.

Please change the work controls on page 3 of our application in section 13.C to contracts.

We have asked for interim funding from the Memorial and Children's Hospital Medical Center Foundation for the period 11/72 through 5/31/73 in the event no funds become available during that time. Final action on this request will be taken by the Foundation Board on October 6, 1972. We expect affirmation on the condition that funding by the Foundation will close if outside support becomes available during the six month period. Assuming the Board approves this request we will have funding through the period necessary for the Council for Tobacco Research - U.S.A. Inc. to consider our request. Dr. Nordsiek indicated this would be March '73, and that an April 1 starting date would be possible. We would like to request that if the application is approved.

Thank you for your attention to this. I will be glad to provide further information if it is needed.

Sincerely,

David M. Mower

David M. Mower, M.D.

1003539080

October 4, 1972

Re: Case No 135 "Effect of Nicotine on the Rat Fetus" 1/1/73 - 12/31/75

Supporting Statement for Application

Justification of budget:

Personnel:

H.D. Mosier, investigator: A \$5,000 salary is requested to relieve Memorial Hospital of Long Beach of this portion of its salary obligation. The amount is approximately 13% of the combined current salary made up by the University base salary and the Memorial Hospital supplement. It does not entirely balance the 40% estimated for the project, but will relieve the investigator of some administrative and teaching obligations and enhance attainment of the project goals.

Regina A. Jansons: Miss Jansons has a B.S. degree and ten year's experience. Seven of these are in the investigator's laboratory in directly related work. The salary is in line with her current salary plus expected merit and cost of living increases anticipated in accord with Memorial Hospital policy. Miss Jansons has considerable experience in management of the animal colony, breeding techniques, measurement in vivo, dissections, homogenizations, extractions, thin layer chromatography, radioisotope determination by scanning, and liquid scintillation techniques, gas chromatography, and miscellaneous biochemical techniques.

Personnel budget provides fringe 6.74% which includes FICA and state of California Workman's Compensation. A 5% increase is calculated for each year of the project to cover anticipated merit or cost of living increases.

Supplies:

Animal care is the largest item but is somewhat less than actual cost. The balance is absorbed by the budget of the Department of Medical Education at Memorial Hospital.

Chemicals and radiochemicals are the next largest item. Radiochemicals are included to provide for the purchase of labelled nicotine to be used in some studies to determine the proportion of nicotine absorbed from diet when it has been mixed with the food in accord with our experiments involving the intake of nicotine throughout pregnancy.

Other expenses:

Equipment maintenance and service contracts: We have sufficient equipment in the laboratory to carry out the present study; however; the cost of maintenance and service contracts for some of the large pieces of equipment

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is a significant item. These pieces of equipment are the Aminco-Bowman spectrophotofluorometer, Hewlett-Packard Model 9100 B programmable calculator, Beckman DU-2 spectrophotometer, and a Packard Instrument Company Model 3320 Liquid Scintillation Counter.

Relevance of this work to research on Tobacco and Health:

Transplacental passage of nicotine has been demonstrated by a number of investigators; early work has been reviewed by Larson et al. (1). Investigations after injection of (-)-nicotine-N-methyl- ^{14}C into pregnant mice have shown the presence of labelled nicotine, cotinine, and other metabolites in fetal tissues (2,3). Evidence has been put forward suggesting that the decidua basalis bars free transplacental diffusion of nicotine (3). In vitro incubation of slices showed no evidence of formation of metabolites by placenta or fetal lung and only traces of cotinine in fetal liver suggesting that fetal tissues had relatively less ability than adult tissues to form metabolites of nicotine (3).

It has been recognized in studies with ^{14}C -nicotine, labelled in the N-methyl group, that demethylated derivatives cannot be detected (2). The advantage of a general label was shown in the experiments of Bowman et al. who were able to show a significant fraction of ^3H -demethylcotinine in the urine of mice injected with ^3H -cotinine (4).

The acute administration of nicotine in pregnant animals has been shown to produce capillary damage in dog placentas (5), teratogenic effects on the skeletal system of the offspring and decrease of litter size in mice (6), vertebral anomalies in chicks (7), and postponement of the appearance of the first litter and lighter weight offspring in rats (8,9). The administration of nicotine to nursing rat mothers is associated with a poor neonatal weight gain and an increase in neonatal mortality (9,10). Geller (11) gave pregnant rats nicotine doses less than 15 percent of those used by Hishimura and Nakai in mice (6) and produced no fetal abnormalities. This difference may be explained on the basis of dosage and species differences.

Both acute and chronic exposure to nicotine have long been known to produce changes in lipid metabolism. Acute administration of nicotine results in a rise in serum free fatty acids (FFA) in dogs and humans (12). Smokers, when compared with non-smokers, have been shown to have increased serum lipoproteins (13) and cholesterol (13-18). However, in some studies there has been no difference observed in cholesterol levels between smoking men in the age range 18-25 (19) and in the age range of 65-86 (20). These differences have not been satisfactorily accounted for.

It is probable that some of the changes in lipid metabolism in the adult are indirectly produced through the nicotine effect on the adrenal medulla causing

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a release of epinephrine (21-23). When epinephrine in oil is administered, there is an immediate lipid mobilization followed in 24 to 48 hours by a rise to peak levels of serum lipoproteins, cholesterol and phospholipids (22-24). These effects may be independent actions of the hormone since the rise of lipoproteins will occur when the FFA response is blocked by glucose or insulin (24). Both responses are abolished by hypophysectomy or adrenalectomy, but can be re-established with cortisone (25). Chronic nicotine intake may produce hyperlipemia in the adult through repeated release of epinephrine and the effect of subsequent repeated rises of FFA on the liver or through some independent action causing a rise in lipoproteins.

Adrenergic blocking agents can inhibit the rise of FFA which result from epinephrine or norepinephrine administration (26). It has recently been shown that the beta adrenergic blocking agents nethalol and a central adrenergic reflex blocking agent, chlorpromazine, are able to block FFA rise after nicotine and inhibit to some extent triglyceride rise. Phenoxybenzamine, an alpha blocker, did not have this action (27). One might infer from this that epinephrine or isopropyl-norepinephrine mediate the release of FFA by nicotine.

A direct nicotine effect in lipid metabolism on the cellular level is implied in recent experiments in dogs in which chronic administration of nicotine resulted in a selective diminution of acetate $1-C^{14}$ incorporation into cholesterol and reduced cholesterol turnover (28). The significance of these effects in overall lipid metabolism remains to be determined.

Smoking during pregnancy in the human has been related to an increased incidence of prematurity and low birth weight infants in a number of studies (29-33). Whether this is a direct effect of tobacco products on fetal growth and the physiology of pregnancy or indirect through such factors as maternal diet cannot be determined with the present data. Our own experiments suggest that maternal dietary disturbance may be one of the elements in the production of low birth weight by tobacco smoking. This conclusion is compatible with recent reports of low birth weight and immaturity of pups of rats given large doses of nicotine during pregnancy. The maternal food intake and weight gain were reduced concomitantly (34).

A number of workers have related smoking or nicotine intake to cardiovascular disorders. A recent review dealt with smoking as a factor in atherosclerosis (35). Chronic administration of nicotine will produce a variety of aortic lesions in adult rabbits. In a recent study, it was shown that there are intimal deposits of mucopolysaccharides and medial lesions with localized necrosis, fibrosis, and calcification (36). When nicotine is administered either intravenously or directly, the microcirculation responds by initial vasoconstriction, then vasodilation. Arteriolar walls thicken (37). Rabbits who have remained without disease after receiving definite doses of vitamin D and dietary cholesterol for many months respond to the addition of nicotine with fatal calcific arterial lesions especially conspicuous in peripheral arteries (38). There medial calcific degeneration of arteries is accompanied

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by fibrocellular proliferation of the media. The mesenchymal reaction attracts xanthomatous accumulations which appear in the thickening intima at serum cholesterol levels no greater than found commonly in man. The changes also occur in cardiac muscle arteries. A recent study showed that subendothelial fibrosis in rabbit arteries after chronic nicotine dosage is inhibited by administration of a mono amine oxidase inhibitor. The authors propose the hypothesis that the monoamine oxidase inhibitor inhibits the action of epinephrine in the production of the arterial lesions.

Generalized calcific arterial disease has long been noted in human infants (39). The etiology is unknown and the disorder is mentioned here only to indicate that generalized arteriopathy occurs in the human infant. Many observations have been made in this condition which have been summarized in two textbooks of pediatric pathology (40,41) and reviewed recently in tow case reports (42, 43). A detailed study in one of these (43) brought out evidence suggesting that mediocalcinosis in infants is a primary elastic tissue disorder with accumulations of mucoid followed by calcification. The intimal fibrosis is seen as a secondary change.

The pharmacology of nicotine has been extensively studied (1). Recent work has been summarized in the Fourth International Symposium at the Wenner Gren Center, Stockholm, 1964 (44). In this, the work of various investigators of the metabolism of nicotine in tissues was brought together. Nicotine injected into the mouse is rapidly accumulated in brain, adrenal medulla and superior cervical ganglion. The accumulation in the brain disappears in 30-60 minutes (45,46). In the rabbit liver 8 metabolites of nicotine have been demonstrated after in vitro or in vivo exposure (47, 48). Both rat and rabbit excrete a mixture of pyridine compounds in the urine. Cotinine is common to both rat and rabbit urine; 8 pyridine compounds have been isolated from rate urine after nicotine (49). In the mouse, nicotine is rapidly metabolized to cotinine in tissues and in one study $C^{14}O_2$ was the only other radioactive compound after administration of C^{14} nicotine. A number of metabolic products were identified by chromatographic methods (50,51).

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51. Hansson, E., and Schmitterlow, C.G., pp. 87-97 (in work cited as ref. 44).

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UNIVERSITY OF CALIFORNIA, IRVINE

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SANTA BARBARA • SANTA CRUZ

DEPARTMENT OF PEDIATRICS
CALIFORNIA COLLEGE OF MEDICINE

IRVINE, CALIFORNIA 92664

Please Reply to:

MEMORIAL HOSPITAL OF LONG BEACH
2801 ATLANTIC AVENUE
LONG BEACH, CALIFORNIA 90801
(213) 595-3236

September 18, 1972

Re: Case No. 135

Robert C. Hockett, Ph.D.
Acting Scientific Director
The Council for Tobacco Research - U.S.A., Inc.
110 East 59th Street
New York, New York 10022

Dear Dr. Hockett:

In response to your letter to September 5, 1972, advising me of the Council's invitation to submit an application for support of our research, I am herewith submitting our formal application for \$92,226 for support of our project "Effect of Nicotine on the Rat Fetus" over a three year period from January 1, 1973 to December 31, 1975.

I have followed the format of the Council for Tobacco Research in preparing the application. There are no sections for budget justifications or for statements of background and rationale of the the project and I have accordingly left these out of the publication. If further information is needed I will be glad to supply it.

The application includes appendices giving expansions of sections 8, 9, 11, and 12. I have already sent you a copy of the letter from the National Institute of Child Health and Human Development advising me of Council approval of our renewal grant in March 1972. We have received no further word on the funding of this grant since I last wrote you. I am enclosing a copy of our paper on the pharmacology of nicotine in the fetus. You have received other copies with my earlier query. This paper has been accepted for publication in Teratology.

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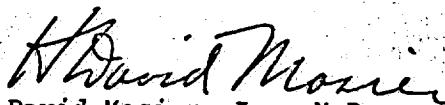
Robert C. Hockett, M.D.

September 18, 1972

Because of our funding crisis for this project I would greatly appreciate the earliest possible funding date if this application is approved by the Council for Tobacco Research, U.S.A., Inc.

I greatly appreciate your kind interest in our work.

Sincerely yours,



H. David Mosier, Jr., M.D.

HDM/rrh

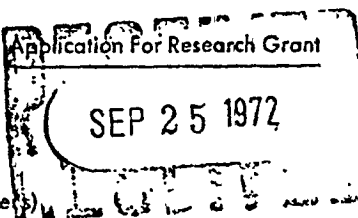
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THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

COMMITTEE:

Dr. Andervont
Dr. Gardner
Dr. Meier

110 EAST 59TH STREET
NEW YORK, N. Y. 10022



Date: September 18, 1972

1. Name of Investigator(s): (include Title and Degree)
H. David Mosier, Jr., M.D. Professor of Pediatrics

2. Institution &

Address: Memorial Hospital Medical Center
2801 Atlantic Avenue
Long Beach, California 90801

3. Short Title of Project:

Effect of Nicotine on the Rat Fetus

4. Proposed Starting Date: January 1, 1973

5. Anticipated Duration of this Specific Study: Three years.

6. Brief Description of Objectives or Specific Aims:

- a. To determine the metabolic effects of high nicotine levels in the fetus and the role of adrenal medullary function in nicotine effects on lipid metabolism in the fetus.
- b. To determine the effect of exposure to high levels of nicotine throughout gestation on the fetal heart, blood vessels, and other organs, and,
- c. To determine the effect of exposure to high nicotine levels during fetal life on postnatal growth and development.

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7. Give a Brief Statement of your Working Hypothesis:

In the adult, nicotine causes a release of epinephrine by the adrenal medulla which in turn promotes fat mobilization. Our tentative hypothesis is that the same reaction occurs in the fetus.

8. Details of Experimental Design and Procedures: (Attach Separate Pages)

See attached sheets

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

See attached sheets

10. Additional Requirements:

None

11. Biographical sketches of all principal and professional personnel (append)

See attached sheets

12. List of publications: (Five most recent as pertinent) (append)

See attached sheets

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12. Budget (1st year)

A. Salaries (Personnel by names)

Professional

H.D. Mosier

includes fringe benefit

% time

Amount

40%

R

Technical

Regina A. Jansons

includes fringe benefit

100%

REDACTED

Sub-Total

REDACTED

REDACTED

B. Consumable Supplies (list by categories)

Animals and animal care	1,750
Chemicals & radiochemicals	1,000
Glassware	500
Photography and art work	300
Misc.	500

Sub-Total

4,050

4,050

C. Other Expenses (itemize)

Costs of publication	600
Histology at 2.50/slide	300
Equipment maintenance and service controls	1000

Sub-Total

1,900

1,900

D. Permanent Equipment (itemize)

Misc.	500
-------	-----

500

E. Overhead (15% of A + B + C)

Total

3,784

29,513

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	REDACTED	3,500	1,700	1,000	3857	30,567
Year 3		3,500	1,700	1,000	4050	32,052

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature

Director of Project

H. David Mosier, Jr., M.D. Telephone

Signature

Business Officer of the Institution

Mr. Jack Weiblen

Telephone

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Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current

Title of Project	Source	Amount	Duration
Effect of nicotine on the rat fetus	NIH HD 04425-03 Total awarded \$51,167 for 3 years	15,556	to 10/31/72
Same title	Memorial Hospital Foundation	approx \$8,000	11/1/72 - 5/31/72

Pending

Effect of nicotine on the rat fetus	NIH Renewal grant HD 04425-04-05-06 Approved by National Advisory Council March 1972; in "hold" for funding - no indication funding will be carried out.	04 - \$38,021 05 - \$26,847 06 - \$27,239 07 - None	11/1/72 - 10/31/75
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(Amounts approved by Council not funded.)

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8. Details of Experimental Design and Procedures:

a. Work in Progress

1) Effects of nicotine on fetal adrenal medullary function

We have set out to measure adrenal content of catecholamine content following a suggestion of Professor A. Jost (Paris) in a personal communication that this may provide as good information on acute release of epinephrine as measurement of plasma levels in the fetus. We are progressing with work along this line using measurements of epinephrine and norepinephrine in fetal adrenals, fetal whole body, and maternal adrenals and maternal plasma after a single injection of nicotine intraperitoneally in the pregnant rat.

Details of the methods are as follows:

Virgin female rats of the Long-Evans strain (Simonsen Laboratories, Gilroy, California) are mated with males of the same strain. Day 0 gestation is the day of appearance of the vaginal plug. The females are then transferred for the duration of the experiment to hanging wire mesh cages 7 x 7 x 10 inches in size. Temperature in the animal room is maintained at 21.6 - 26.7°C. Lights are set for 14/10 hour light/dark cycle. Purina Lab Chow and tap water are given ad lib. On day 20 of gestation the pregnant rats are given an injection of nicotine 1 mg/kg ip. The solution injected contains 1 mg nicotine per cc sterile isotonic saline. Controls are injected similarly with sterile isotonic saline or are not injected (0 time controls). In a study now in progress the animals consist of three non-injected rats considered at time 0, three saline controls, and three nicotine injected rats sacrificed at 5, 10, 15, 30, 45, and 60 minutes after injection. At sacrifice the pregnant rat is decapitated. Neck blood is collected in chilled centrifuge tubes containing 0.2 ml heparin sulfate 1:5000 and 5 mg sodium metabisulfite. The tubes are swirled gently and placed in ice. The abdomen and uterus are rapidly opened, fetuses removed, stripped of membranes, and separated from placentas, quickly swirled in a bath of isotonic saline to clean of blood and amniotic fluid, snapped in perforated plastic capsules and dropped into liquid nitrogen. The maternal adrenals are then removed and put into plastic capsules and then liquid nitrogen. The tissues are removed to pre-chilled screw capped jars and held at -60°C for dissections. The maternal plasma is separated in a refrigerated centrifuge and frozen in vials at -60°C pending determinations.

The procedure for determination of epinephrine and norepinephrine follows the method of Anton and Sayre, J. Pharmacol. Exp. Therap. 138:360, 1962. We have recently modified this to include final re-acidification after conversion to the lutine. The determinations of fetal adrenals and maternal adrenals given in the table below were done with this modification. For dissections of fetal adrenals, the fetuses are allowed to thaw at room temperature and the adrenals are removed with aid of a stereo-zoom dissection microscope. All peri-adrenal fat is removed. Three pairs of adrenals from fetuses of the same maternal rat are combined for each determination. Each pair of maternal adrenals are weighed and combined for determination. The results of fetal adrenal determinations are given as μg per fetus. The fetal adrenals were not weighed

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in this study in order not to delay the procedure and risk degradation of the catechols. Weights of fetal adrenals at 20 days gestation in this strain of animals have been determined in our laboratory previously. These average 2.2 mg for males and 1.7 for females. No attempt was made to sex the fetuses in this series as it seemed unlikely that adrenal medullary volume itself would have a corresponding sex difference.

The results to date of this study are given in the figures a and b and in the descriptive paragraphs below.

An examination of the data indicates that the epinephrine content of fetal adrenals 30 and 45 minutes after nicotine injection is significantly lower than that of the controls. In controls there is an upward trend of fetal adrenal content from 0 time value by 45 minutes. This could be the result of handling and maternal excitement caused by the injection. Nicotine apparently eliminates this rise in adrenal concentration.

Norepinephrine concentration in the fetal adrenal is somewhat more variable. No significant difference is seen between nicotine and saline injected animals. Both, however, appear to have an increase in norepinephrine concentration evident from 10 minutes through 45 minutes. Normal values are regained by 60 minutes.

Maternal adrenals after nicotine injection show a marked drop in epinephrine concentration between 5 and 10 minutes. Norepinephrine changes little after nicotine injection by 10 minutes. Saline controls had a marked drop in epinephrine concentration by 5 minutes. Values from 15 minutes through 60 minutes have shown no differences between nicotine and saline injected animals. This work is preliminary and requires confirmation.

Fetal whole body concentrations of epinephrine and norepinephrine at 15, 30, 45, and 60 minutes show no differences between nicotine and saline injected rats. Determinations are yet to be carried out on 5 and 10 minute specimens.

Maternal blood has not yet been assayed.

This work is still in progress. The results thus far suggest an immediate (5 minutes) reaction of maternal medullary discharge of epinephrine content after nicotine injection. Fetal adrenal response is delayed and seems to consist mainly of a lack of the progressive increase in epinephrine content observed in the saline injected groups. Work is planned to repeat earlier time periods with a larger dose of nicotine, e.g. 5 mg/kg, in order to determine whether the fetal adrenal response may be dose related. This increase in dose should more than compensate for the difference in fetal and maternal plasma concentration. Our earlier studies (see Mosier and Jansons, article in press, Teratology, attached) showed that 5 minutes after iv injection of labelled

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nicotine in maternal rat, the radioactivity concentration in fetal plasma was more than half that of maternal plasma and the proportion of nicotine to metabolites was greater in fetal than maternal plasma. We may carry out a comparison study with iv injections, under pentobarbital anesthesia. This should avoid the portal circulation on the first circulatory passage of nicotine and enhance delivery of the dose to the fetus. Possible interference of the barbiturate at the level of the fetal adrenal medulla will be considered in interpreting results.

2) Effects of administration of nicotine throughout pregnancy on fetal plasma, lipid phosphorus, and cholesterol.

Nicotine is added to finely ground Purina Lab Chow to give concentrations of 0.05 mg/g or 0.10 mg/g. From day 0 or from days 10-11 in gestation to day 20 the nicotine diet is given in place. Control animals are maintained on finely ground Purina Lab Chow. Tap water is given ad lib. Food intake and body weight at intervals of 2-3 days are recorded. At day 20 animals are anesthetized lightly by ether fumes, the abdomen is quickly opened and fetuses removed from their membranes. They are quickly blotted, decapitated, and bled into heparinized capillary tubes. The plasma is separated quickly by centrifugation in the cold, and stored at -60°C pending analysis.

a. Lipid phosphorus

Determinations are carried out on 40 microliter quantities of plasma by a modification of the method of Zilversmit and Davis, J. Clin. Lab. Med. 35:155, 1950. In a preliminary study carried out during summer of 1972 the following results were obtained:

Nicotine concentration in diet mg/g	N	Lipid Phosphorus (mg/100 ml) Mean \pm SEM
0.05 days 10-20	4	5.648 \pm 0.1540
0.10 days 0-20	5	6.079 \pm 0.2113
0.10 days 10-20	4	5.246 \pm 0.1248
controls	4	5.471 \pm 0.4548

Plasma of each litter was pooled; duplicate determinations were carried out per pool. Results of the litters were averaged.

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b. Cholesterol

Plasma from the same series used in the lipid phosphorus study described above is in storage awaiting cholesterol determinations by a micromodification of the method of Henry, Clinical Chemistry, 1964. pp. 856-858.

b. Work Planned

1) Acute effect of nicotine on fetal plasma non-esterified fatty acids (NEFA) and triglycerides.

Pregnant rats on day 20 of gestation will be injected with 1 mg/kg nicotine and sacrificed with fetal bleeding as described above at 10, 20, and 30 minutes after injection. Non-injected (0 time) and saline injected controls will be included. This series is being collected at present. The fetal plasma NEFA will be determined by an adaptation of the method of Elphick, J. Clin. Path. 21:567, 1968. Triglycerides will be determined by an adaptation of the method of Soloni, Clin. Chem. 17:529, 1971.

2) Effect of adrenergic blocking agents on response of fetal NEFA and triglycerides to nicotine.

Groups of pregnant rats at 20 days gestation will be injected intraperitoneally according to one of the following schedules: (a) isotonic saline, two injections one hour apart, or (b) isotonic saline followed in one hour by nicotine 0.1 mg/kg; or (c) an adrenergic blocking agent (see table below) followed in one hour by nicotine 0.1 mg/kg.

TABLE

Blocking Agent	Dose	Receptor Blocked
Phenoxybenzamine	8 mg/kg	Alpha
Pronethalol	5 mg/kg	Beta
Chlorpromazine	5 mg/kg	Central adrenergic reflex

The doses of nicotine and blocking agents will be changed if preliminary measurement of the effect and data of a), above, indicate this would be desirable. At 10, 20, and 30 minutes (with separate groups for each time interval) the abdomen will be opened and the fetuses quickly removed and exsanguinated by methods described above. Determinations will be carried out in duplicate in litter pools of plasma.

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- c. Effect of fetal nicotine exposure on cardiovascular renal systems after birth.

Litters of rats exposed to different concentrations of nicotine and controls will be killed at different ages from birth through 121 days of age to determine whether gross or microscopic alterations can be seen in heart, vessels, or kidneys. Weights of organs including heart, kidneys, gonads, and adrenal glands will be measured at these autopsies for comparison with controls.

- d. Effect of fetal nicotine exposure on subsequent growth

Litters of pregnant nicotine fed rats and control rats will be observed for 121 days after birth. Nicotine will be stopped at day 20 of gestation. Litters will be cut to 8 pups per mother. Body weight and tail length will be measured at weekly intervals beginning at two days of age. At 121 days, at sacrifice, tibial length and tibial epiphyseal width will be measured.

Figures a. and b.

- a. epinephrine and norepinephrine content of fetal adrenals
b. epinephrine and norepinephrine content of fetal whole body

9. Physical facilities available

Office and laboratory - located in the Earl and Loraine Miller Children's Hospital in the Memorial Hospital Medical Center of Long Beach. These include:

Investigator's office
Secretary's office
Biochemical laboratory, bench space, usual outlets, sinks, fume hood.
Room for heavy equipment
Room for sensitive instruments
Storage and equipment room
Room for trainees, calculator, photography, and files
Animal facility with room for animals with controlled air circulation, temperature, lighting, surgical room laboratory, and instrument room; storage room.

Access to certain special facilities in the four-story clinical laboratory wing of Memorial Hospital under the direction of Elmer R. Jennings, M.D. These include also glass washing service provided by the staff of the laboratory.

Animal caretaking is provided by the Department of Medical Education of Memorial Hospital in exchange for a set day rate per animal.

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-6-

Access to a Control Data 3300 computer facility for specifically approved research requests.

Special equipment now in the investigator's laboratory:

Cahn electrobalance, Mettler semi-micro balance, Roller-Smith torsion balance, Packard Tri Carb Model 3320 liquid scintillation counter and auto-gamma accessory, Packard Model 7201 Radio-chromatogram scanner, IEC Model PR-2 refrigerated centrifuge, Revco ultra deep freezer, Aminco-Bowman spectro-photofluorometer with X-Y recorder, Beckman Model DU-2 direct reading spectrophotometer with Digital Concentration Converter and Linear-Log Recorder; Polaroid MP-3 copier; Hewlett-Packard Model 9100 B program table calculator with X-Y Recorder and Printer.

11. Biographical sketch of investigator:

Personal:

REDACTED

Education: B.S. University of Notre Dame, Notre Dame Indiana
M.D. The Johns Hopkins University School of Medicine,
Baltimore, Maryland

Internship: Pediatrics, The Johns Hopkins Hospital

1952-3

Residency: Assistant Resident in Pediatrics, Los Angeles Children's
Hospital
Resident in Pediatric Pathology, Los Angeles Children's
Hospital

1953-4

1954-5

Fellowship: Fellow in Pediatric Endocrinology, The Johns Hopkins
Hospital, Baltimore, Maryland

1955-7

Certification: Certified by the American Board of Pediatrics

1957

Licensure: Maryland Board of Examiners

1952

California Board of Medical Examiners

1958

Career: Assistant in Pathology, University of Southern California
Assistant Professor of Pediatrics, University of California,
Los Angeles

1954-5

1957-61

Associate Professor of Pediatrics, University of
California, Los Angeles

1961-3

Consultant in Endocrinology, Pacific State Hospital,
Pomona, California

1957-63

Director of Research, Illinois State Pediatric Institute,
Chicago, Illinois

1963-7

Associate Professor of Pediatrics, University of Illinois
College of Medicine, Chicago, Illinois

1963-7

Senior Consultant, Los Angeles County General Hospital

1967-8

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Physician in Charge, Pediatric Endocrine Clinic, Cedars-Sinai
Medical Center, Los Angeles

1967-8

Professor of Pediatrics, University of California, Irvine

1967-

12. Selected publications:

Richter, C.P. and Mosier, H.D., Jr.: Maximum sodium chloride intake and thirst in domesticated and wild Norway rats. Am. J. Physiol. 17:213, 1954.

Mosier, H.D., Jr.: The development of the hypothalamoneurohypophyseal secretory system in the chick embryo. Endocrinology 57:661, 1955.

Mosier, H.D., Jr.: Comparative histological study of the adrenal cortex of wild and domesticated Norway rats. Endocrinology 60:460, 1957.

Mosier, H.D., Jr. and Blizzard, E.M.: Defects in the biosynthesis of thyroid hormone in congenital goitrous cretinism. A.M.A.J. Dis. Child. 94:390, 1957. (Abstract).

Blizzard, R.M. and Mosier, H.D., Jr.: Protein binding properties of mono- and diiodotyrosine as compared with thyroxine and triiodothyronine. A.M.A.J. Dis. Child. 94:534, 1957. (Abstract)

Mosier, H.D., Jr., Blizzard, R.M., and Wilkins, L.: Congenital defects in the biosynthesis of thyroid hormone. Pediatrics 21:248, 1958.

Mosier, H.D., Jr., and Richter, C.P.: Response of the glomerulosa layer of the adrenal gland of wild and domesticated Norway rats to low and high salt diets. Endocrinology 62:268, 1958.

Mosier, H.D., and Armstrong, M.K.: Absence of binding of inorganic iodide I^{131} by irradiated human serum protein in vitro. Endocrinology 11:671, 1962.

Mosier, H.D., Jr., Armstrong, M.K., and Schultz, M.A.: Measurement of the early uptake of radioactive iodine I^{131} by the thyroid gland: A method requiring reduced irradiation. Pediatrics 31:426, 1963.

Mosier, H.D., Jr., and Armstrong, M.K.: Effects of maternal intake of nicotine on fetal and newborn rats. Proc. Soc. Exp. Biol. & Med. 116:956, 1964.

Mosier, H.D., Jr.: Presence of the long-acting thyroid stimulator in serum in mongolism without hyperthyroidism. J. Clin. Endocr. 25:1005, 1965.

Mosier, H.D., Jr., Grossman, H. J., and Dingman, R.F.: Physical growth in mental defectives. Pediatrics, suppl., 36:465, 1965.

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- Mosier, H.D., Jr.: In vitro incubation of rat and rabbit thyroid with L-tyrosine- C^{14} sodium iodide- I^{131} and L-mono- and diiodotyrosine- C^{14} - I^{131} . Proc. Soc. Exp. Biol. & Med. 121:573, 1966.
- Dingman, H.F., Mosier, H.D., Jr., and Grossman, H.J.: Deviation in somatic growth: a factor analysis. Child Development 27:949, 1966.
- Mosier, H.D., Jr., and Armstrong, M.K.: Effect of maternal nicotine intake on fetal weight and length in rats. Proc. Soc. Exp. Biol. & Med. 124:1135, 1967.
- Mosier, H.D., Jr. and Jansons, R.A.: Stunted growth in rats following X-irradiation of the head. Growth 31:139, 1967
- Mosier, H.D., Jr., and Richter, C.P.: Histologic and physiologic comparisons of the thyroid gland of the wild and domesticated Norway rat. Anat. Rec. 158:263, 1967.
- Mosier, H.D., Jr.: The neck-thigh radioiodine ratio in mongolism. J. Ment. Defic. Res. 2:97, 1967.
- Mosier, H.D., Jr., and Jansons, R.A.: Pituitary content of somatotropin, gonadotropin, and thyrotropin in rats with stunted linear growth following head x-irradiation. Proc. Soc. Exp. Biol. & Med. 128:23, 1968.
- Mosier, H.D., Jr.: Thyroid function in mongolism: Evidence for a defect in iodide trapping. Proceedings of XII International Congress of Pediatrics, Mexico City, Dec., 1968. (Abstract)
- Mosier, H.D., Jr.: Allometry of body weight and tail length in studies of catch-up growth in rats. Growth 33:319, 1969.
- Mosier, H.D., Jr.: Effect of X-irradiation on selected areas of the head of the newborn rat on growth. Radiat. Res. 43:92, July 1970.
- Mosier, H.D., Jr.: Causes of failure of catch-up growth after certain forms of growth retardation. Pediat. Res. 4:459, 1970.
- Mosier, H.D., Jr.: Failure of compensatory (catch-up) growth in the rat. Pediat. Res. 5:59, 1971.
- Mosier, H.D., Jr.: Transplacental passage of nicotine in the rat. Clin. Res. 19:222, 1971. (Abstract)
- Mosier, H.D., Jr., and Jansons, R.A.: Transplacental passage of nicotine in the rat. Proceedings of the XIII International Congress of Pediatrics, Vienna, Austria, August 1971. (Abstract).
- Mosier, H.D., Jr., and Jansons, R.A.: Allometry of body weight and tail length after head X-irradiation in rats. Growth 35:23, 1971.

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Mosier, H.D., Jr.: Decreased energy efficiency after cortisone induced growth arrest. Growth 36:123, 1972.

Mosier, H.D., Jr., Smith, F.G., and Schultz, M.A.: Failure of catch-up growth after Cushing's syndrome in childhood. A.M.A.J. Dis. Child. 124:251, 1972.

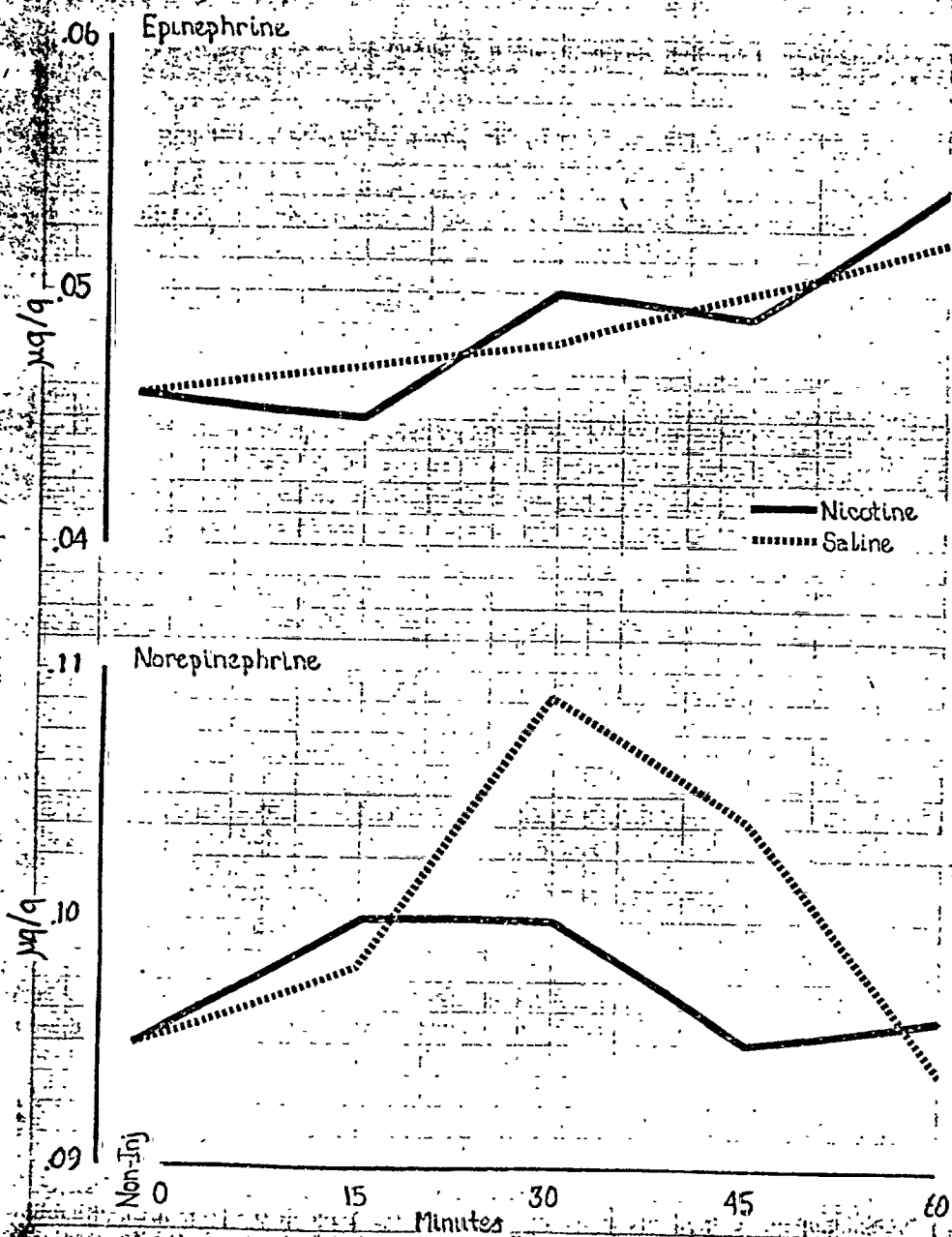
Mosier, H.D., Jr., and Jansons, R.A.: Distribution and fate of nicotine in the rat fetus. Teratology (In press).

Dearden, L.C., and Mosier, H.D., Jr.: Hypothyroidism induced by propylthiouracil and its effect on the ultrastructure of tibial epiphyseal chondrocytes during long term recovery. Anat. Rec. (In press).

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Fig. 3

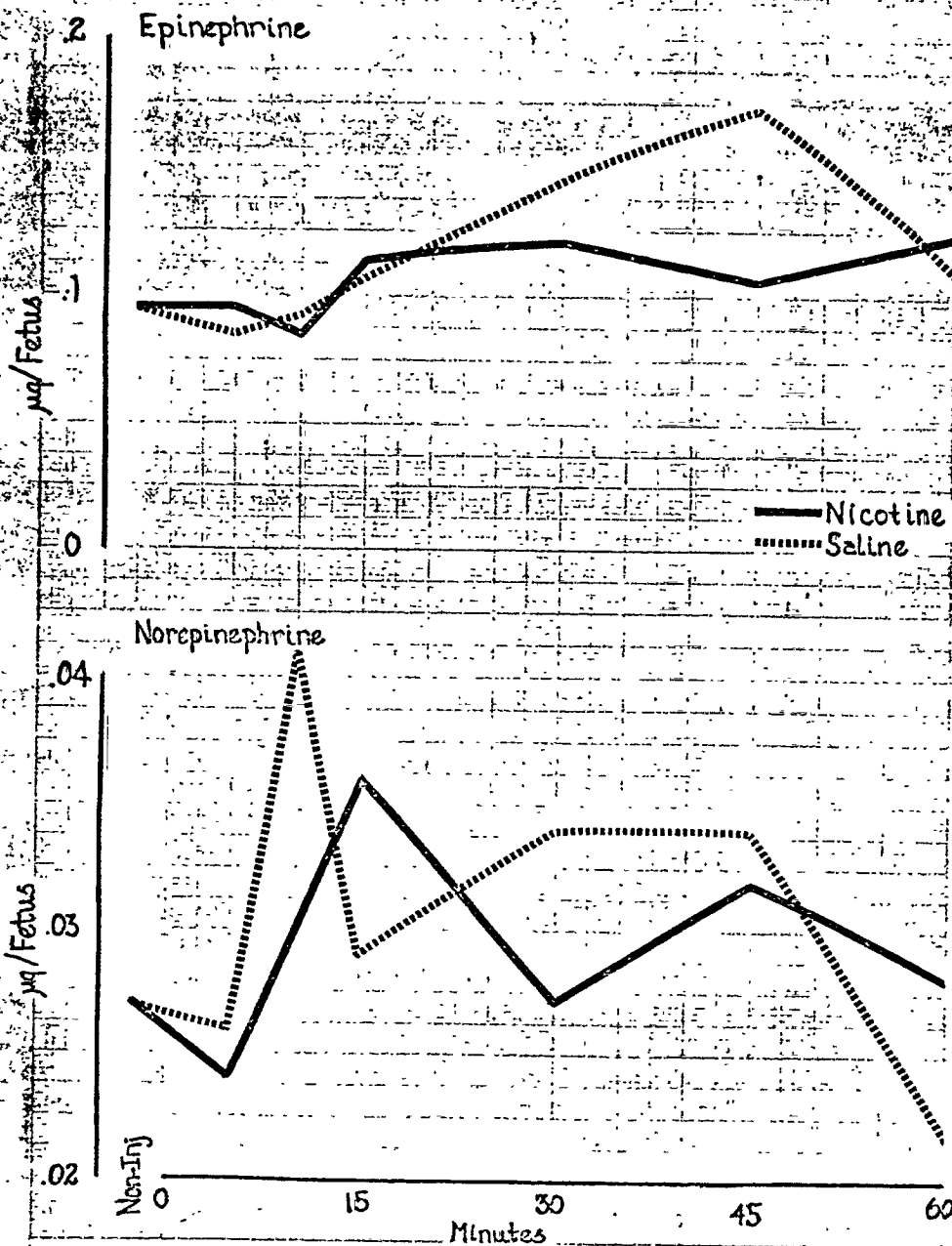
FETAL WHOLE BODY



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Fig. A.

FETAL ADRENALS



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ACCOPRESS

NO. 2507

BF-FED	BL-GOLD
BG-BLACK	BQ-PALM GREEN
BO-LT GREY	BA-EXECUTIVE RED
BP-LT GREEN	BR-BROWN
BULT-ELLE	BA-TANGERINE
BY-YELLOW	BB-ROYAL BLUE
SPECIFY NO. & COLOR CODE	

4000 DIVISION OF GARY INDUSTRIES, INC.
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